

# Molecular Regulation of Bud Phenology and Vegetative Phase Change in *Populus* Trees

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Doctoral thesis

Swedish University of Agricultural Sciences

Umeå 2020

Acta Universitatis Agriculturae Sueciae  
2020:3

Cover: *Populus tremula x tremuloides* wild type and transgenic plants.

ISSN 1652-6880

ISBN (print version) 978-91-7760-524-9

ISBN (electronic version) 978-91-7760-525-6

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Print: Original Tryckeri, Umeå 2020

# Molecular Regulation of Bud Phenology and Vegetative Phase Change in *Populus* Trees

## Abstract

Trees are sessile organisms that have evolved to survive and thrive in changing growth conditions. As a survival strategy they undergo massive morphological changes that can be quantified. Post germination, during the transition from juvenility to maturity, many plants undergo well defined phase changes, for instance in vegetative morphology and in the capacity of the plant to produce reproductive organs. These phases reflect underlying changes in gene regulation within the plants. Along with aging, trees being perennials have to survive across several years being exposed to seasonal cycles. Extreme winter conditions make it particularly difficult for trees to survive due to cold induced damages and drought. Trees sense the coming of the winter and cease the growth of their meristematic tissues and undergo bud formation. This too is under the control of an underlying genetic machinery. Earlier studies on the annual weed *Arabidopsis thaliana* has already uncovered the co-ordination between changing environmental conditions and the changes in the cellular machinery it triggers. In this thesis, I have used this already available knowledge and studied the effects of the aging related miR156 and miR172 genes in regulating phase change and apical bud phenology in the model tree *Populus tremula* x *tremuloides*. In addition to that I have also studied the function of the *GIGANTEA* gene in the same photoperiodically regulated control of growth cessation and bud set.

**Keywords:** juvenile to reproductive phase shift, growth cessation, bud set, bud burst, photoperiod, aging, circadian clock, *miR156*, *miR172*, *GIGANTEA*.

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## Molekylär reglering av knoppfenologi och vegetativ fasförändring hos hybridasp (*Populus tremula* x *tremuloides*).

### Sammanfattning

Träd är fast rotade och orörliga organismer som har utvecklats för att överleva och växa under väldigt varierande klimatförutsättningar. Som en del av deras överlevnadsstrategi så genomgår de stora morfologiska och kvantifierbara förändringar. Efter groning genomgår många växter, under övergången från juvenilitet till mognad, väl definierade fasförändringar, t.ex. i den vegetative tillväxten och i förmågan att reproducera sig. Dessa fasförändringar styrs av en underliggande differentiell genreglering. Träd som växer i tempererade och boreala delar av världen måste, samtidigt som de åldras och eftersom de är perenner, överleva många år av klimatiska säsongsvariationer. Extrema vinterförhållanden gör det extra svårt för träd att överleva på grund av risken för köld och torkskador. Träd kan förutsäga när vintern kommer och kan som en anpassning sluta växa och sätta knopp, detta styrs också av en underliggande genreglering. Forskning på den ettåriga modellväxten *Arabidopsis thaliana* har redan gett oss stora insikter om koordinationen mellan förändrade miljöförhållanden och de cellulära och molekylära förändringar som dessa inducerar. I denna avhandling har jag utgått från denna kunskap och har studerat hur två åldersrelaterade mikroRNA, miR156 och miR172, kontrollerar vegetativ fasförändring och knoppsättningsfenologi hos modellträdet hybridasp (*Populus tremula* x *tremuloides*). Jag har också studerat funktionen hos genen *GIGANTEA* i samma dagslängdsstyrda reglering av knoppsättningstiden

**Keywords:** juvenilitet, den vegetative tillväxten, förmågan reproducera mikroRNA, miR156, miR172, *Arabidopsis thaliana*, *Populus tremula* x *tremuloides*, *GIGANTEA*.

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# Dedication

To Aai, Baba and Prajakta...

ॐ (OM)

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## List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Ding, J., Böhlenius, H., Ruhl, M.G., Chen, P., Sane, S., Zambrano, J.A., Zheng, B., Eriksson, M.E., and Nilsson, O (2018). *GIGANTEA*-like genes control seasonal growth cessation in *Populus*. *New Phytologist*, vol (218), pp. 1491-1503.
- II Sane, S\*, Klintonäs, M\*, Chen, P., and Nilsson, O (2020). miR156 affects the juvenile to adult transition and the timing of bud set in Poplar trees. (Manuscript)
- III Sane, S., Klintonäs, M., Ding, J., and Nilsson, O (2020). Role of miR172 and its targets in the regulation of bud phenology in Poplar (manuscript)

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\* These authors contributed equally to this paper

The contribution of Shashank Sane (SS) to the papers included in this thesis was as follows:

### **Paper 1**

-SS performed a part of the practical work and contributed to the paper.

### **Paper 2**

-SS and Maria Klintenäs planned, executed the project and wrote most of the manuscript along with Ove Nilsson.

### **Paper 3**

-SS planned the experiments with ON. SS executed most of experiments and wrote the manuscript along with Ove Nilsson.

## Abbreviations

35S	Cauliflower mosaic virus 35S promoter
ABA	Abscisic acid
ABI1,3	ABSCISIC ACID INSENSITIVE1,3
AG	AGAMOUS
AIL	AINTEGUMENTA-LIKE1
AP1-2	APETELA1-2
AP2L	APETELA2-LIKE
BFT	BROTHER OF FT AND TFL1
BiFC	Bimolecular fluorescence complementation assay
BR	BRASSINOSTEROIDS
CAL	CAULIFLOWER
CALS1	CALLOSE SYNTHASE1
CCA1	CIRCADIAN CLOCK ASSOCIATED
CDF	CYCLIN DOF FACTOR
CDL	critical day length
CEN	CENTRORADIALIS (TFL ortholog)
ChIP	chromatin immunoprecipitation
CK	CYTOKININS
CO	CONSTANS
Col	<i>Arabidopsis thaliana</i> Columbia ecotype
COL1	CORONATINE-INSENSITIVE PROTEIN
CRY	CRYPTOCHROMES
DAM	DORMANCY ASSOCIATED MADS
DNA	Deoxyribonucleic acid
EBB	EARLY BUD BREAK
EE	evening element

ELF	EARLY FLOWERING
ET	ETHYLENE
Fig.	figure
FKF1	FLAVINBINDING KELCH REPEAT F BOX PROTEIN 1
FLC	FLOWERING LOCUS C
FRI	FRIGIDA
FT	FLOWERING LOCUS T
ft	<i>Arabidopsis FT</i> mutant
FUL	FRUITFULL
GA2,20 OX	Gibberellin 2,20-oxidase
GI	GIGANTEA
GID1	GIBBERELLIN INSENSITIVE DWARF1
GIL	GIGANTEA-LIKE
JA	JASMONATE
JAZ	JASMONATE-ZIM
kDa	kilo Dalton
LAP2	LIKE-APETELA2
LDP	Long day plant
LFY	LEAFY
LHY1,2	LATE ELONGATED HYPOCOTYL
LKP2	LOV KELCH PROTEIN2
LUX	LUX ARRHYTHMO
ME	morning element
MFT	MOTHER OF FT AND TFL1
MIM	MIMIC
miR	microRNA
miRNA	microRNA
NO	NITRIC OXIDE
nt	nucleotides
ORF	Open reading frame
PEBP	phosphatidylethanolamine-binding protein
Pfr	active far-red
PHYA-E	PHYTOCHROME A-E
PIF4	PHYTOCHROME INTERACTING FACTOR4
Pr	in-active red
PRR3,5,7,9	PSEUDO-RESPONSE REGULATOR 3,5,7,9
Pt	<i>Populus trichocarpa</i>
Q-PCR	quantitative- polymerase chain reaction
RISC	RNA induced silencing complex

RNA	ribonucleic acid
SA	SALICYCLIC ACID
SAM	shoot apical meristem
SBP	SQUAMOSA-PROMOTER BINDING PROTEIN
SDP	short day plant
SLY	SLEEPY
SMZ	SCHLAFMÜTZE
SNZ	SCHNARCHZAPFEN
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1
SPA	SUPPRESSOR OF PHYA
SPL	SQUAMOSA PROMOTER BINDING PROTEIN LIKE
SVL	SHORT VEGETATIVE PHASE-LIKE
SVP	SHORT VEGETATIVE PHASE
T6P	TREHALOSE-6-PHOSPHATE
T89	<i>Populus tremula x tremuloides</i> male clone T89
TOC1	TIMING OF CAB 1
TOE1-3	TARGET OF EAT 1-3
TOL1-4	TARGET OF EAT-LIKE1-4
UTR	untranslated region
UV	ultra violet
WT	WILD TYPE
ZT	ZEITGEBER TIME
ZTL	ZEITLUPE

# 1 Introduction

Plants are sessile organisms that cannot relocate in order to survive. Environmental conditions like change in photoperiod, temperature, water and nutrient availability has forced plants to be morphologically flexible and adapt for survival. This is seen in the various phenotypic changes that the plants undergo over its life cycle. The vegetative phase change, the juvenility-to-maturity transition, reproductive phase, time of flowering and fruit bearing, shade avoidance and drought resistance are examples of such adaptations. All these traits have been observed and studied by generations of agriculturist and botanist in order to improve crop production and to quench their thirst for knowledge of the plant world. As human population grows and our demand from nature grows it has become vital to study this primary resource. In this thesis, I have studied aspects of the juvenility-to-maturity transition, vegetative phase change and bud set phenology in the model genus *Populus* (Poplar).

## 1.1 Why study Poplar as a model tree?

Forestry and allied industries are vital contributors to the Swedish economy. Study on trees to improve their traits for commercial purpose and sustainable forestry has been of interests to researches for decades. Traditional breeding for elite genotypes has been the tool of choice until the advent of molecular biology. Presently, with genome sequencing and various other molecular biology tools developed over years we have enhanced our ability to study at depths that was not possible before. The annual flowering *Arabidopsis thaliana* genome was sequenced in 2000 (Arabidopsis Genome, 2000). Since then we have extracted a lot of information on developmental pathways and genes in this model plant (Aukerman and Sakai, 2003). Since all plants are evolutionarily related and their fundamental mechanisms conserved, it has made study of other plant species

easier. Since *Arabidopsis* is a small herb with lack of extensive secondary growth and an annual life cycle which is different from trees, it is obvious that it is important to complement the *Arabidopsis* research with the study of trees. Trees are perennial organisms with a strong trunk supporting a foliage. The secondary growth by cork and vascular cambium, ability to survive in inclement weather over years, phenotypic flexibility due to dormancy establishment, cold hardening, bud formation and juvenile and reproductive phase shift has allowed it to be a dominant growth strategy in the plant kingdom. The Poplar tree is a deciduous, fast growing, easy to manage angiosperm. It has a modest genome size and a range of genetic tools are already available, e.g., its transformation with *Agrobacterium tumefaciens* is routinely established, making it an ideal model tree for the genome sequencing and to study by reverse genetics.

## 1.2 The Poplar tree

The name Poplar originates from the Latin “*Populus*” meaning people, since the trees were planted in public spaces in ancient times. Poplar is an angiosperm *Eudicot* from the *Rosid* clade, order *Malpighiales* and family *Salicaceae*. There are around 22 species of Poplar (Zhou et al., 2018). They are deciduous trees that grow in the entire northern hemisphere. The size of Poplar trees depends on the species and varies between 15 to 60 meters in height and up to 3 meters in diameter (DeBell, 1990). The tree bark can be white, light green, brownish or grey in colour. Depending on the age of the tree it can be either smooth or with deep ridges. It has a substantial root diameter and is very invasive and destructive near construction sites. Majority of the species are dioecious i.e.; they have male and female flowers on separate trees. As the spring arrives flowering takes place before leafing. Individual flowers are crowded on the catkin that appears from an inflorescence bud break event in spring (Eckenwalder, 1996). Poplar pollens are wind pollinated and the seeds are dispersed by wind as they are covered with cotton tufts of silky hair (Slavov et al., 2009). Size and shape of leaves depends on the species and the age of the tree. They are generally oval or heart shape, with serrated margins. Aspen trees (*Populus tremula* and *Populus tremuloides*) have flattened petioles that tumble in the wind. At Umeå Plant Science Centre we use hybrid aspen (*Populus tremula* x *tremuloides*) as a model tree, a clone originating from the Czech Republic called “T89”, as it is relatively easy growing and relatively easy to transform. Post germination, Poplar has two distinct phases in its life cycle, the juvenile phase when they do not flower in permissive conditions, and the reproductive phase when they flower. Poplar elite



lines under special care are able to flower in 5 years but generally most species flower in 12 to 15 years (Slavov and Zhelev, 2010).

### 1.3 Poplar genome

The Poplar genome is 50 times smaller and thus relatively compact in comparison to gymnosperms like Spruce making it an excellent choice as a model tree for genome sequencing. It has 19 chromosomes and is 4 times larger than *Arabidopsis thaliana*, the first plant genome to be sequenced. There have been two whole genome duplication events during the evolution of *Populus* (Grigoriev et al., 2012). Due to a whole genome duplication, chromosomal duplications and tandem gene arrangements, Poplar makes a good model for comparative studies with *Arabidopsis thaliana* (Tuskan et al., 2006, Nordberg et al., 2014).

### 1.4 Phenotypic changes from juvenile to reproductive phase in trees

Plants undergo several developmental transitions during its life cycle. The first is germination and transition of the embryo within the seed into a post-embryonic structure. The second being the post-embryonic phase with development into a juvenile vegetative plant. Eventually, the third and final adult reproductive phase follows in which plants flower and bear fruit in permissible environmental conditions. The juvenile phase lasts for a short period in annual plants while in perennials it lasts for years. This makes the study of phase change much more visual in perennial trees. The juvenile to adult vegetative phase change is usually morphologically visible by differences in leaf shape and size, the number of internodes and height of the stem, presence or absence of trichomes and cuticular wax on the leaf surface. Juvenile and adult vegetative phase is also visible in a spatial-temporal manner on plants, with older branches having juvenile characteristics while the younger more apical branches bearing adult vegetative characteristics (Wang et al., 2011).

### 1.5 Annual and perennial lifestyles

Plants that complete their life cycle within a single season i.e.; that germinate, grow vegetatively, flower and set fruit, undergo senescence and death within a seasonal cycle are called annuals e.g. *Arabidopsis thaliana*. *Arabidopsis* due to

its short life cycle was one of the first models selected for study on plant molecular genetics. Plants that survive over multiple seasonal cycles are called perennials. In order to survive over long years perennials have evolved several unique features. They undergo growth cessation, bud set, dormancy establishment and hardening to survive in inclement weather. They have longer juvenile phases in order to establish themselves before channelling their energies into reproduction (Hyun et al., 2017). They have both vegetative meristems and floral meristems and have dedicated shoots programmed to a determinate outcome. Perennials have a distinct phenotype in juvenile and adult phases. This phenotypic metamorphosis probably equips them with advantages that result in long multi-seasonal survivability. Phase change in trees was first described in *Acacia* species that is native to Australia, where it is characterised by dramatic differences in leaf morphology (Wang et al., 2011). In juvenile phase this species produces horizontally oriented, bipinnately compound leaves. Later, its transition to adult phase is marked by production of phyllodes i.e., vertically oriented simple leaves in which adaxial cell types are present on both surfaces of the leaf blade. Different *Acacia* species have this transition at different nodes, which is accompanied by a gradual production of transition leaves with both leaf types being present on a single leaf. Juvenile and adult stages of vegetative development are also well differentiated in many other species like *Quercus acutissima*, *Hedera helix*, *Acacia confuse*, *Acacia colei*, *Eucalyptus globulus* etc (Wang et al., 2011).

## 1.6 Short day and Long day plants

Plants flower in a suitable season to maximise their chance for pollination, seed production and dispersal. For this, plants have evolved and specialised in accordance to the environment they are exposed to for maximum outcome. Photoperiod is the most variable cue in terms of latitudinal location and is also the most recurrent cue for the sessile plants. Flowering time in boreal and temperate habitats differs from flowering in tropical and equatorial habitats. Maximum productivity at various latitudes must coincide with maximum germination, vegetative growth and survivability. Plants at higher latitudes are at the risk of being exposed to freezing temperatures if the flowering takes place in early autumn. On the other hand, flowering too early in the summer can be disastrous for tropical grasses like *Oryza sativa* (Rice) that requires puddled water for growth. Plants have evolved to manage this by specialising for different regions. In boreal regions they flower when the day length is longer than the critical length, thus being called Long Day Plants (LDP). In contrast,

Short Day plants (SDP) flower below a certain threshold day length (Andres and Coupland, 2012, Song et al., 2015). Plants that do not use that cue at all are called Day Neutral Plants, e.g. tomatoes. Critical Day Length (CDL) by definition is the minimum light period in a cyclical 24<sup>hrs</sup> scale that the plant requires above or below which it is able to flower. The CDL is regulated by two pathways that interact to generate this phenology i.e.; the Photoperiodic pathway and the Circadian Clock. In it, the photoreceptors like phytochromes and cryptochromes interact with the circadian clock proteins like CIRCADIAN CLOCK ASSOCIATED1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY) and TIMING OF CAB EXPRESSION1 (TOC1) (Song et al., 2015). Plants in long nights (darkness) on exposure to light pulses at certain diurnal timepoints are able to produce flowers revealing interaction between photoperiod and the circadian clock (Song et al., 2015). *Arabidopsis thaliana* is a facultative LDP, since it flowers faster at longer day lengths but flowers non-the-less in short days (SD). *Populus* is a tree that grows in boreal habitat and flowers in the spring as the day lengthens and thus can be considered as a LD plant in terms of flowering. However, in terms of growth cessation and bud set in the fall, *Populus* trees behave as SD plants, responding to a shortening of day length. The T89 clone of *Populus tremula* x *tremuloides* that we use for this study has a critical day length of 15.5<sup>hrs</sup> (Olsen. et al., 1997).

## 1.7 Flowering time in *Arabidopsis thaliana*

*Arabidopsis* is an annual weed from the *Rosid* clade and family *Brassicaceae*. Its natural habitat is from the arctic circle to Cape Verde near the equator (Baurle and Dean, 2006). The environment in this region has four seasons due to change in day length over the year. *Arabidopsis* has thus adjusted to this scenario by flowering in the optimum seasonal window to produce maximum vegetative growth, pollination and seed dispersal. This period is identified by long days and warmer temperatures. Thus, *Arabidopsis* falls in the category of facultative long day plants i.e.; it will flower faster at day lengths which are long but can flower even in short days although much later (Kobayashi and Weigel, 2007). There are various other environmental and internal cues that are responsible for the flowering that are being discussed below in detail.

### 1.7.1 Photoperiod

Since temperature fluctuates during the day and can vary considerably between years it is difficult for many plants to make long term decisions that impact their

survival based on this cue. Instead they use photoperiod as signalling cue to trigger events in their life cycle. Day length is the most dependable recurrent signal since the length of the day at a particular date of the year will always be the same. In 1920s, it was first suggested that plants use quantitative day length as a cue for flowering (Garner. and Allard., 1920). As the field developed further it became clear that day length triggers a factor in the leaves of the plants that is transported to the shoot apex to trigger flowering. In 1936 the Soviet scientist Chailakhyan suggested the Florigen hypothesis (Zeevaart, 2006). The Florigen that promoted flowering was later identified as the FT protein. It was later re-named as FLOWERING LOCUS T (Kobayashi and Weigel, 2007, Kardailsky et al., 1999). Grafting experiments showed that FT was indeed produced in the leaves and later transferred to the shoot apical meristem SAM to trigger flowering. The change in day length and light quality is sensed in the leaves by five phytochrome receptors PHYA, PHYB, PHYC, PHYD and PHYE along with two cryptochromes CRY1, CRY2 and phototropins. A CONSTANS - FLOWERING-LOCUS-T(CO-FT) module is central to the photoperiodic regulation of flowering in Arabidopsis (Suarez-Loapez. et al., 2001). It was also found to be a highly conserved mechanism in the photoperiodic pathways in multiple species within the Angiosperm lineage. The CO protein is expressed twice in the day, once in the morning and late during dusk (Song et al., 2015, Imaizumi, 2010). Various repressors like CYCLIN DOF FACTOR (CDF) proteins degrade CO and thus controls its expression (Fornara et al., 2009). This degradation is overcome by the concurrent expression of GIGANTEA (GI) and FLAVIN BINDING-KELCH REPEAT-F BOX1 (FKF1) that stabilises the CO expression when exposed to light. Blue light receptor proteins like CRY2 affect expression of the circadian clock regulated proteins GI and their partner FKF1 forming a dimeric ubiquitin ligase complex and stabilising the expression of the diurnally expressed CO (Sawa et al., 2007). In long days, when the light exposure is above the critical day length (CDL) of the plant, the CO expressed between afternoon to night coincides with light and is stabilised. This stabilised CO is able to bind the *FT* promoter and trigger its expression (Corbesier et al., 2007). FT in turn acts as a Florigen and is transported from the leaves via the phloem to the apical meristem triggering flowering (Jaeger and Wigge, 2007, Mathieu et al., 2007). Apart from the *CO-FT* regulon, the hormone gibberellin (GA) is also involved in photoperiod regulated flowering in Arabidopsis. But GA and the *CO-FT* regulon act as largely parallel pathways as lack of one does not stop plants from flowering (Eriksson et al., 2015). GA levels are low in SD but increase in LD. But it is the GA levels that triggers flowering in SD when the *CO-FT* regulon is inactive (Wilson et al., 1992).

### 1.7.2 Vernalization

Although photoperiod is central to flowering being triggered in Arabidopsis, the day length is the same twice in a year at the same dates. Once in the spring solstice and other in autumn solstice. This can cause confusion, a problem solved by using multiple environment cues, vernalization is one such process. There are two types of genotypes in Arabidopsis, the winter annuals and the summer annuals. The winter annuals cannot flower unless they are exposed to chilling winter temperatures for a certain period of time. They grow vegetatively over summer and flower in the next spring. Winter annuals are found at higher latitudes where growing seasons are very short (Gazzani et al., 2003, Michaels et al., 2003). Growing vegetatively in the autumn and lying dormant gives it an advantage to flower and set seeds in the coming short spring. The summer annuals germinate, grow vegetatively, flower and die in the same growing season, the ecotype Columbia (Col) is one such variety. The difference in lifestyles of these plants provides them with maximum survivability. The winter annual life cycle is due to the presence of a functional MADS-box *FLOWERING LOCUS C (FLC)* gene which encodes a repressor of *FT* expression during vegetative growth preventing flowering. *FLC* is activated by an active *FRIGIDA (FRI)* gene in Arabidopsis (Michaels et al., 2004). The *FLC* gene is downregulated when plants are exposed to chilling temperatures in the winter. The memory of *FLC* downregulation is maintained over the arrival of long day and warm temperatures in the spring (Bergonzi et al., 2013, Romera-Branchat et al., 2014). The genetic memory of downregulation is dependent on epigenetic factors (Amasino, 2004, Simpson and Dean, 2002). The summer annuals that have an inactive *FRI* or *FLC* gene consequently flower and set seeds in the same season without the need for chilling (Amasino and Michaels, 2010).

### 1.7.3 Circadian clock

The circadian clock is an endogenous timekeeper that operates in all organisms with a period of approximately 24<sup>hrs</sup>. Its genetic components and interactions are extensively conserved in plants, bryophytes and blue green algae suggesting its common evolution and functional importance (Harmer, 2009). The presence of a circadian clock mechanism is important to keep a cyclical expression of genes and metabolites to anticipate and integrate environmental changes due to change in photoperiod and temperature across day-night cycles and seasons. An automatic circadian clock mechanism provides disciplined, cyclical inputs to the organism and is the foundation of its internal organisation. This adds to the fitness and robustness of the cellular machinery (Millar, 2016). A wide range of

processes like movement of leaves, stem elongation, stomatal opening and closing, metabolic processes like photorespiration and photosynthesis are regulated by the clock (Imaizumi, 2010). It also regulates seasonal phase changes, flowering and bud phenology. Thus, a broken circadian clock mechanism due to mutation in its vital component genes can be fatal for the organism (Yanovsky and Kay, 2002). The vital components of the circadian clock consist of various connected internal feedback loops that positively or negatively regulate each other. It consists primarily of morning- and evening-expressed clock genes. The Morning Element (ME) consists of two MYB-transcription factors i.e.; CIRCADIAN CLOCK ASSOCIATED1(CCA1), LATE ELONGATED HYPOCOTYL (LHY) and the Evening Element (EE) consists of the PRR-family member TIMING OF CAB EXPRESSION1 (TOC1)(Song et al., 2015). The morning element is highly expressed from dawn to afternoon and it negatively regulates the evening element *TOC1*. *TOC1* in turn is a positive regulator of *CCA1* and *LHY* and triggers their expression at dawn. There are other associated proteins of the PSEUDO-RESPONSE REGULATOR (PRR) family proteins, notably PRR5 and PRR6, that also play a vital role in promoting and repressing various morning and evening elements making a web of feedback loops (Shim and Imaizumi, 2015). Mutations in any one of these genes breaks the web of pathways that run the oscillation of the clock and is manifested by changes in period and amplitude of the clock. This change in period and amplitude of the clock depending on the mutation produces phenotypical changes in the plant and its interaction with the environmental inputs. The circadian clock gene network works in tandem with photoperiodic pathway elements like PHYTOCHROME and CRYPTOCHROME photoreceptors, CONSTANS (CO), GIAGANTEA (GI), ZEITLUPE (ZTL), FLAVIN-BINDING KELCH REPEAT F-BOX1 (FKF1), CYCLIN DOF FACTOR (CDF) *etc* (Sawa et al., 2007). It also interacts with the vernalization pathway and *FT* repressors like the MADS-box proteins FLOWERING LOCUS C (FLC) and SHORT VEGETATIVE PHASE (SVP).

#### 1.7.4 Aging

*Arabidopsis* plants are unable to flower despite being in environmental conditions conducive for flowering unless they have gone through an age-related juvenile-to-adult phase transition. Each phase has its unique phenotypic characteristics with juvenile plants having small rosette leaves with no abaxial trichomes and smooth margins while, in older plants, as they gain biomass, the leaves are much larger in size and greener with serrated margins and hairy

trichomes. The plants undergo bolting with cauline leaves and eventually produce an inflorescence flowering meristem, forming flower meristems on its flanks that develop into flowers (Huijser and Schmid, 2011, Poethig, 2010). Two microRNA families have been implicated in this age-related change, miR156 and miR172. In *Arabidopsis*, miR156 contains a family of 8 subtypes from miR156a to miR156e. They also have a paralog in miR157 that is 21 nucleotide long in comparison to 20 nucleotides of miR156 and is expressed in some tissues (Kozomara and Griffiths-Jones, 2011, Yang et al., 2013, Yu et al., 2013). miR156 is expressed in all tissues of the plant and is highly expressed during the earlier period of its life cycle (Axtell and Bartel, 2005, Xie et al., 2005). Expression studies have shown that miR156 expression is high in seeds and juvenile plants. When overexpressed the plants are moderately late flowering with a juvenile phenotype retained on a large number of leaves. miR156 targets the SBP domain-containing *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* genes (Rhoades. et al., 2002). There are 17 *SPL* genes in *Arabidopsis* out of which 11 have target sites for miR156. There are four classes of *SPL* genes that have been characterised according to their size and number of domains. The most studied and important are two classes consisting of *SPL3*, *SPL4*, and *SPL5* in one class and *SPL9* and *SPL15* in the second class (Schwarz et al., 2008, Wang et al., 2009). *SPL3*, *SPL4* and *SPL5* encode smaller proteins in comparison to other *SPLs* and is transcribed with two exons. The complementary sites for miR156 on the *SPL* gene transcripts are either within the Open Reading Frame (ORF) or in the 3'-Untranslated region (UTR). In case of *SPL3*, *SPL4*, *SPL5* the target site is found in the 3' - UTR (Gandikota et al., 2007). When miR156-resistant (r) forms of *SPL3*, *SPL4* and *SPL5* are overexpressed from the CaMV 35S promoter the plants flower earlier than the WT (Yamaguchi et al., 2009, Wu and Poethig, 2006, Wang et al., 2009). Immunoprecipitation studies have shown that *SPL3*, 4 and 5 bind to the promoter sequences of inflorescence meristem identity genes like *LEAFY (LFY)*, *FRUITFULL (FUL)* and *APETELA1 (API)* (Yamaguchi et al., 2009). The second group consisting of *SPL9* and *SPL15* are larger proteins and when overexpressed effect the vegetative phase of the plants. *SPL9* over-expression have faster growth initiation with abaxial trichomes on the leaf displaying an adult vegetative phenotype in comparison to WT. *SPL9* is known to induce the expression of the miR172b transcript and thus also induces the adult phase of the plant (Wu et al., 2009). Although *SPL9* and *SPL15* belong to the same group they have slightly different spatial expression profiles with *SPL9* being primarily expressed in leaves and weakly in apical meristem flanks before floral transition. In contrast, *SPL15* is expressed in the vegetative meristem before induction and

later during floral induction and in the inflorescence meristem (Hyun et al., 2016). The *Arabidopsis spl9 spl15* double mutants are severely late flowering in SD while they are slightly late flowering in LD (Hyun et al., 2016, Schwarz et al., 2008). SPL9 and SPL15 over-expression lines flower much earlier than WT displaying adult morphological traits (Wang et al., 2009, Wu et al., 2009). The other SPLs also have minor redundant roles with the previously discussed two main groups (Xie et al., 2010). The target site decides the accessibility of the miR156-RNA induced silencing complex (RISC) (Wang et al., 2009, Yamaguchi et al., 2009). Since there is redundancy in the function of *SPL* genes, single gene mutants do not produce significant phenotypic change in plants. Double or triple or quadruple mutants are required to determine gene function. Recent studies on the *Brassicaceae Arabis alpina* accession Pajares has thrown light on the role played by age determining miR156 on vernalization dependent flowering in this perennial species. The study showed that a flowering response of *Arabis alpina* plants is dependent on the downregulation of miR156 as the plants get old (Bergonzi et al., 2013). Younger juvenile plants with higher expression of miR156 are not sensitive to vernalization and fail to flower while target mimicry-induced sequestration of miR156 results in flowering of young vernalized plants. In contrast, overexpression of miR156 in older plants delays vernalization-induced flowering. This effect is opposite to the effect in *Arabidopsis thaliana* where overexpression of miR156 causes late flowering only in SD and young juvenile plants in LD flower irrespective of miR156 expression as photoperiod plays a dominant role in this species (Bergonzi et al., 2013). As the plants grow older the level of miR172 expression increases. miR172 targets the *APETALA2* (*AP2*)-like transcription factor gene family including *TARGET OF EAT1* (*TOE1*), *TOE2*, *TOE3*, *SCHLAFMUTZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*) and *AP2* (Chen, 2004). Overexpression of miR172 results in earlier flowering in SD with a downregulation of the target genes while a target mimicry line delays flowering (Yant et al., 2010, Jung et al., 2007, Franco-Zorrilla et al., 2007, Todesco et al., 2010). When a miR172 resistant version of the *TOE1* gene was overexpressed, it caused delayed flowering (Mathieu et al., 2009). It was later shown that the *AP2* domain containing genes directly bind the *FT* promoter region acting as a repressor (Mathieu et al., 2009). Both *miR172* and *AP2* family genes are also involved in the determination of floral architecture (Bowman et al., 1991) acting in the outer whorls of the flower (Aukerman and Sakai, 2003, Chen, 2004). It is still a mystery as to how the age regulates expression of miR156 in the first place. Some light has been thrown on this issue by observations that plants grown on sugar-free media stayed juvenile for a longer time while supplementing the media with sugars accelerated



the adult phenotype. Since sugar production and concentration is photosynthesis dependent, plants having a defective photosynthetic machinery stay juvenile for a longer time. Even pruning the leaves delays vegetative phase and flowering as the miR156 expression in such plants is higher (Hyun et al., 2017). Some studies point towards a correlation of H3K27me3 methylation, a chromatin mark associated with the repression of transcription, and the accumulation of sugars in plants (Xu et al., 2016, Xu et al., 2018). A further series of genetic experiments also implicated another sugar, trehalose 6-phosphate (T6P), as a repressor of miR156 levels (Wahl et al., 2013, Hyun et al., 2017). This area is still understudied but has interesting outcomes in agriculture.

### 1.7.5 Hormonal pathways

Hormones are plant metabolites that are ubiquitously distributed and are known to play an important role in various developmental processes. Amongst the well-known and studied hormones are Gibberellins (GAs), Absciscic acid (ABA), Jasmonate (JA), Brassinosteroids (BRs), Cytokinins (CKs), Ethylene (ET), Salicylic acid (SA) and Nitric oxide (NO) (Davis, 2009, Kazan and Lyons, 2016). GA is a key hormone known to play a role in plant growth and development and in Arabidopsis is known to be essential for numerous developmental processes like seed germination, elongation and flowering (Achard and Genschik, 2009). In Arabidopsis plants, the actions of the GA pathway in flowering has been well studied. Overexpression of the GA biosynthesis enzyme GA20 oxidase results in early flowering in both LD and SD, while expression of the GA catabolic enzyme GA2 oxidase results in non-flowering plants in SD (Galvao et al., 2012, Conti, 2017). In LD, GA biosynthesis mutants can still flower as the photoperiod regulated CO-FT module plays a more central role in triggering the expression of downstream targets (Wilson et al., 1992). Since the CO-FT module is non-functional in SD, the plants are then dependent on GA signalling to modulate the expression of downstream floral meristem identity MADS-box genes like *SOC1*, *FUL*, *API* and *LFY* (Hou et al., 2014, Lee and Lee, 2010). GA is primarily found in the leaf tissues and in the apical meristem where it triggers formation of floral meristems. Exogenous application of GA to plants also triggers flowering in SD (Wilson et al., 1992, Porri et al., 2012). The mode of action of GA is very complex as GA homeostasis has to be maintained to trigger developmental process at the correct time. GA signalling is primarily regulated by a class of five nuclear proteins called DELLA proteins (Harberd, 2003, Achard et al., 2007). The DELLA proteins bind to and act as repressors of various downstream elements. The GA-

DELLA homeostasis with various GA and DELLA subtypes and their distribution and regulation of various downstream elements makes studies difficult (Daviere and Achard, 2016, Xu et al., 2014). GA degrades DELLA via a ubiquitin-proteasome system (Griffiths et al., 2007). The proteolytic pathway is initiated with GA binding to a soluble receptor called GIBBERELLIN INSENSITIVE DWARF1 (GID1) triggering a conformational change in the receptor. The modified receptor with increased affinity for DELLA later along with another stimulated E3 Ubiquitin ligase SLEEPY1 (SLY1) causes DELLA degradation (Dill et al., 2004). So, while mutant DELLA lines result in early flowering the mutants *gid1*, *ga* or *sly1* cause late flowering in Arabidopsis (Porri et al., 2012, Galvao et al., 2012). The DELLA proteins play a subtle role in regulating transcription factor genes from parallel flowering pathways. It is known to interfere with activity of the transcription factor CO, thus a higher expression of DELLA results in lower *FT* expression (Tiwari et al., 2010). It also plays a role in the degradation of *SPL* genes (particularly *SPL9* and *SPL15*) in Arabidopsis, thus in-turn lowering the expression of miR172 and consequently upregulating its target AP2 domain containing genes (Yu et al., 2012). As mentioned above, AP2 domain containing genes are repressors of *FT* expression in Arabidopsis thus resulting in late flowering. Similarly, DELLAs interfere with the binding of the PHYTOCHROME INTERACTING FACTOR4 (PIF4) transcription factor to the *FT* promoter, thereby preventing activation (Daviere and Achard, 2016). Besides GA, other hormones also play a role in flowering, but to a lesser extent. The involvement of ABA in flowering is still conflicting as it acts as both repressor and promoter of flowering in Arabidopsis. In Arabidopsis ABA acts as a positive regulator of flowering in LD via its upregulation of *FT* and *TSF* expression, while ABA mutants delay flowering in LD (Riboni et al., 2014). The flowering defect of ABA mutants are not seen in SD. In contrast to that, ABA also plays a role as a negative regulator of flowering via its activity downstream of FT. This negative role on flowering is through its interaction with the MADS -box flowering meristem identity gene *SOC1* (Wang et al., 2016). Such contrary effects on regulating flowering call for more studies in this area. Other hormones that are involved in regulation of flowering are jasmonates (JAs), that are fatty acid derived molecules. Central to JA regulation is JASMONATE-ZIM (JAZ) a family of transcription factor repressors whose degradation is regulated by the F-box protein CORONATINE-INSENSITIVE PROTEIN1 (COL1) (Chini et al., 2007, Conti, 2017). JA binds to the two proteins resulting in the degradation of the JAZ proteins. JAZ acts by preventing the activity of transcriptional factors that modulate JA response. The *coll* mutant lines are early flowering in both LD and SD while overexpression of a JA-

nondegradable form of JAZ also results in early flowering supporting the role of JA signalling in flowering. It does this by upregulating FT by an indirect mechanism.(Conti, 2017, Zhai et al., 2015). The JAZ proteins bind to and repress the AP2-like transcription factor genes *TOE1* and *TOE2*, thus promoting *FT* expression which results in flowering. JAZ activity is also modulated by the activity of GA. DELLA binds to JAZ and disrupts its function resulting in the de-repression of the expression of the AP2-like *FT* repressors causing late flowering. Additionally, DELLA also downregulates miR172 levels resulting in the compounding of AP2 de-repression resulting in late flowering (Conti, 2017, Yu et al., 2015). Brassinosteroids (BRs) act as flowering promoters as the BR mutants are late flowering. BRs act as epigenetic modulators of the *FLC* locus and BR mutants display a higher expression of *FLC* (Domagalska et al., 2010, Domagalska et al., 2007, Li et al., 2010). Ethylene act as a flowering repressor in Arabidopsis and cause late flowering in both LD and SD. This is seen when plants are directly applied with ethylene or the ethylene synthesizing genes are constitutively activated (Achard et al., 2007, Achard et al., 2006). NO and SA have contrasting effects on Arabidopsis flowering, as NO acts as a repressor while SA acts as a promoter of flowering. CKs promote flowering as application of CKs to Arabidopsis plants promotes flowering in SD (Conti, 2017). Since miR172 and its target AP2-like transcription factor genes cross talk with the hormonal pathway genes it would be interesting to study this aspect in our *Populus* plants in the future.

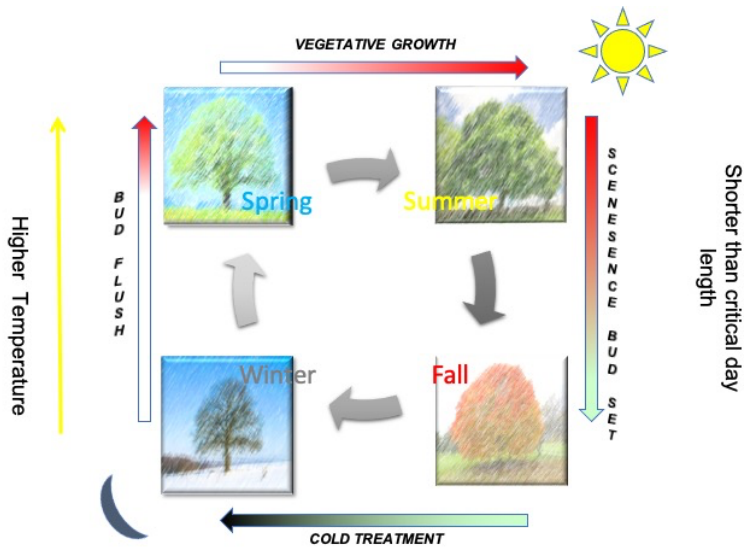
## 1.8 Comparative study of Arabidopsis and Poplar

With the advent of genome sequencing and computational tools to analyse the data collected, it was realised that there is a lot of evolutionary similarity in inheritance and function of genes. These genes were divided based on certain criteria into orthologous and paralogous genes. Orthologous genes are homologous genes that are derived from a common ancestor and are seen amongst different species, they may or may not have a common function. Paralogs are homologous genes that are derived by a gene duplication event within a species but might have evolved different functions. Genetic pathways are also similar between species. This means that comparative studies of divergent plant species are possible if the sequenced genomes are available. The most detailed study was conducted on *Arabidopsis thaliana* which paved the way for a deep study of the tree Poplar. Both being angiosperms gave more fidelity to the studies. One example is the discovery that the CO-FT module

plays a vital role in both flowering and bud phenology regulation (Bohlenius et al., 2006)

## 1.9 Short day induced growth cessation in trees

Since trees have to survive over inclement weather conditions like extreme cold and drought during winters, they arrest their growth. This growth arrest is a temporary feature as long as the environmental conditions are not permissible for growth. During this period, it becomes absolutely crucial to protect tissue from harsh cold and dry conditions. The shoot apical meristem (SAM) undergoes a process of growth cessation, bud formation, dormancy establishment and establishment of cold hardiness. There are several exogenous and endogenous factors that regulate this process. The exogenous factors is decrease in the day length and temperature as autumn sets in and the endogenous factor is modulation of genes that are required to maintain vegetative growth. As the day length falls below the critical day length (CDL) i.e., below 15.5 hrs in *Populus tremula x tremulodes* (T89) plants, the leaves where this signal is sensed triggers a process of growth cessation. With the day length shorter than the critical day length, leaf primordia formed after the SD shift, arrests growth and form the embryonic leaf, while the cuticle form scale like structures that enclose the SAM to form a closed bud structure (Rohde and Bhalerao, 2007). As the day length and temperature reduces further in autumn, the apical buds that are formed accumulate phenylpropanoids and other metabolites and turn reddish brown in colour. In most trees a shortening of photoperiod triggers this process. In SD, *FT2* transcription ceases and the tree induces preventive measures to protect itself from damages caused by advent of winter. A downregulated *FT* expression further acts on apical meristem genes like *LAPI*, *AIL* and eventually cell cycle genes like D-type cyclins that play a role in cell division thus ceasing growth (Karlberg et al., 2011, Azeez et al., 2014). Interestingly, there are trees like apple and pear that do not use photoperiod and rather use low temperatures to trigger this process (Tanino et al., 2010, Olsen et al., 2014). Following is a description of our current understanding of mechanisms that regulate apical growth cessation in trees, particularly in *Populus*.

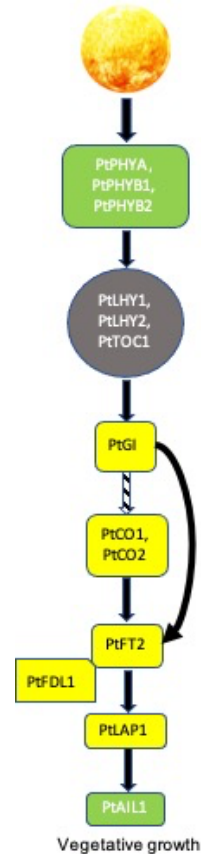


**Figure 1:** Life cycle of a tree in boreal habitat.

### *Photoperiodic pathway*

Plants use day length to measure seasonal changes as it is the most consistent cue to regulate its developmental processes. This sensitivity is thanks to the plethora of leaf-based receptor proteins like phytochromes, cryptochromes, phototropins and other light sensitive proteins like ZEITLUPE, LUX, FKF1 *etc.* There are two types of phytochromes in *Populus* i.e., PHYA and PHYB (Ingvarsson et al., 2006). Phytochromes are photochromic proteins that are found in photo-interconvertible isomeric forms i.e., in-active Red (Pr) and active Far-Red (Pfr) form. On exposure to red light the Pr form becomes activated into the Pfr form with an activated nuclear homing signal driving the Pfr form to the nucleus (Song et al., 2015). In the autumn, as the day length shortens and with extended periods of Far-Red-enriched twilight in the morning and afternoons, the balance shifts towards the inactive Pr form which cumulates in the regulation of different developmental processes like flowering and bud set. This light sensing by the receptors provide inputs to the diurnally expressed circadian clock oscillator genes resulting in the entrenchment of the endogenous developmental pathways with the environmental conditions. Significant amongst the peripheral genes connected to the circadian oscillator are *GI* and *CO* whose diurnal expression pattern is modulated by both the photoperiod and circadian clock. The diurnal oscillation of *CO* expression has a major peak at the end of the day around ZT 14 to ZT 16 and is central to *FT* regulation (Bohlenius et al., 2006).

This module of CO-FT regulation is consistent with the external coincidence model that was proposed in 1936 first by Bünning and later elaborated by (Pittendrigh and Minis, 1964). The diurnally expressed CO protein is only stable under incidental light at the end of the LD when CO protein is upregulated as without incidence of light CO is degraded by various repressors. The CO protein binds to a unique cis-region proximal to the *FT* promoter and positively regulates its expression in long days (Tiwari et al., 2010). This FT protein produced in the vascular bundles of the leaves and is then transferred to the apex via the phloem to trigger developmental processes at the apical meristems. A high *FT* expression is a repressor of *Populus* bud set as it results in transgenic plants growing even in SD (Bohlenius et al., 2006). Once in the shoot apical meristem FT acts along with its interactors like the transcription factor FDL1. This complex later regulates downstream elements like LAP1 and AIL1 that positively regulate cell cycle genes in the apical meristem, thereby powering vegetative growth (Franklin, 2008, Azeez et al., 2014, Tylewicz et al., 2015).



**Figure 2:** Photoperiodic pathway in *Populus*

### *Components of the photoperiodic pathway in trees*

There are various modules within the photoperiodic pathway that act together to direct bud phenology in trees. These modules are photoreceptors, diurnally operational genes, promoters and repressor of FT and apical meristem identity genes. All these modules apart from regulating the photoperiodic pathways are also communicating with other pathways during plant development.

#### Photoreceptors

*Populus* can sense light by the photoreceptors present in the leaves. They consist of phytochromes, cryptochromes, phototropins and unidentified UV-receptors. The most studied amongst the receptors are the Red and Far-Red light sensing phytochromes and the blue light sensing cryptochromes. The PHYA, PHYB1 and PHYB2 receptors have been the subject of several studies. Overexpression

of Oat PHYA in *Populus* results in no growth cessation when plants are exposed to SD conditions (Olsen. et al., 1997), conversely downregulation of PHYA by RNAi resulted in early growth cessation compared to wild type lines (Ibanez et al., 2010). PHYB function remains to be characterized in *Populus*. Cryptochromes are blue -light receptors that play a promotive role in the photoperiodic regulation of FT (Sawa et al., 2007).

### *GIGANTEA*

GIGANTEA is a unique plant specific nuclear protein that plays a central role in integrating the circadian clock output of diurnally expressed genes with external photoperiodic signals. In Arabidopsis, GI protein along with the LIGHT OXYGEN VOLTAGE (LOV) domain containing FLAVIN -BINDING KELCH REPEAT F-BOX1 (FKF1) blue-light receptors degrades the CO repressors. The incidence of blue light generates a GI-FKF1 complex that triggers the ubiquitin dependent degradation of the target CDF family proteins that repress CO expression. The stabilised CO proteins in-turn bind to the promoter of *FT* and triggers its expression (Sawa et al., 2007). GI also physically associates with the regulatory regions of the *FT* gene where repressors like SVP and TEM bind and thus neutralizes their negative effect. The CO-FT module plays a central role in the photoperiodic regulation of flowering in Arabidopsis. Both *FT* and *CO* RNAi lines have a late flowering phenotype in LD (Bohlenius et al., 2006, Ding and Nilsson, 2016). This suggest that *CO* regulation plays a central role in this model. This is not the case with *Populus* where artificially upregulating the *CO* expression, or RNAi-induced downregulation, has no effect on the regulation of *PttFT2* and consequently growth cessation and bud phenology.

### *CONSTANS*

Downstream of the photoreceptors and GI, is the B-box-type zinc finger protein CONSTANS (CO). *CO* is expressed in a diurnal pattern across a 24 hrs time period. In Arabidopsis it peaks at dawn as its expression starts rising in the dusk that broadly coincides with the LD photoperiod resulting in a stable CO protein expression (Suarez-Lopez et al., 2001). Expression analysis of Arabidopsis *CO* orthologs in *Populus* showed that they are expressed robustly in the leaves during the growing season suggesting their regulation by long photoperiod (Bohlenius et al., 2006). There are two *CO* loci in *Populus* i.e; *PtCO1* and *PtCO2*. There is difference in the abundance of *PtCO2* transcript according to the age of the plant as *PtCO2* is highly expressed in mature leaves in comparison to juvenile leaves, while *PtCO1* is expressed equally in juvenile and mature

leaves (Hsu et al., 2012). RNAi down-regulation of *CO* expression results in earlier bud set in Poplar when shifted to day lengths shorter than the critical day length (Bohlenius et al., 2006). Since the critical day length (CDL) to trigger bud set is latitude specific in poplar, genotypes native to southern Sweden set bud later in comparison to genotypes from northern Sweden. The CDL for southern genotypes was much longer as the south had a later advent of winter and a milder winter in comparison to the north where winters arrive early and is longer and much harsher. The diurnal expression peak of *CO* was believed to regulate this variation (Bohlenius et al., 2006). But, since 35S::*CO*-expressing trees respond similarly to wild type plants in photoperiodic shift experiments, it was thought that *CO* was not the only player for bud set regulation by photoperiod (Hsu et al., 2012). This also showed that there are differences in the function of *PtCO1* and *PtCO2* compared to its ortholog in *Arabidopsis*, *CO*. It was recently confirmed that *GIGANTEA* (*GI*) also directly binds the *PttFT2* promoter and regulates its expression (Ding et al., 2018), and it was proposed that *PttGI* has a more important role in bud developmental regulation than *Populus CO*.

### *FT* gene family

Poplar has a family of 20 kDa Phosphatidylethanolamine domain-binding proteins with three *FT*-like genes. They are *PtFT1*, *PtFT2 $\alpha$*  and *PtFT2 $\beta$*  (Wang et al., 2018), *CENTRORADIALIS1* and 2, (*PtCEN1*, *PtCEN2*), *MOTHER OF FT AND TFL1* (*PtMFT*) and *BROTHER OF FT AND TFL1* (*PtBFT*) (Mohamed et al., 2010). Phosphatidylethanolamine domain-binding proteins are found in all taxa i.e., prokaryotic bacteria and eukaryotes (Banfield et al., 1998). Like its *Arabidopsis* counterpart, Poplar *FT* genes appear to be involved in the regulation of flowering as overexpression of both *FT1* and *FT2* cause early flowering (Bohlenius et al., 2006). *FT2* is highly expressed in leaves during the vegetative phase in spring and summer, while *FT1* expression is absent during this period. The *FT1* expression rises in buds during the winters when exposed to low temperatures while *FT2* expression plummets in the fall after the Poplar plants are exposed to short days (Ding and Nilsson, 2016). *PttFT2* is expressed at the end of the day at dusk (Bohlenius et al., 2006) and the photoinduced *CO*-*FT* regulon modulates growth cessation in Poplar. This module is a component of the photoperiod pathway with light and circadian clock related components like *GI* controlling its expression (Ding and Nilsson, 2016, Ding et al., 2018). The overexpression of both *FT1* and *FT2* from the CaMV 35S promoter acted against SD-induced growth cessation, while RNAi downregulation led to early cessation and bud set (Bohlenius et al. 2006). Like *Arabidopsis FT*, Poplar *FT2* was shown to positively regulate the *APETELA1* tree ortholog *Like-API* (*LAPI*) (Azeez et



al., 2014) through an interaction with the FD-like proteins FD-like1(FDL1) and FD-like2 (FDL2) (Tylewicz et al., 2015). This in turn leads to an activation of the *AINTEGUMENTA-like 1* transcription factor (*AIL1*) (Karlberg et al., 2011, Azeez et al., 2014) and downstream cell cycle genes. Along with *FT* are two *CEN* genes that like their Arabidopsis ortholog *TFL1* encodes repressors and cause early growth cessation and bud set when overexpressed and delays it when being downregulated (Mohamed et al., 2010). *PtMFT* has been shown to not have any effect on bud phenology but it is speculated that it might play a role in Poplar seeds (Mohamed et al., 2010).

#### *Like-AP1 (LAP1)*

In Arabidopsis *APETALA1* (*AP1*) is a MADS-box transcription factor that is involved in flowering and is highly similar to other Arabidopsis floral meristem identity genes like *FRUITFULL* (*FUL*) and *CAULIFLOWER* (*CAL*) (Kaufmann et al., 2010, McCarthy et al., 2015). The *Populus* ortholog called *Like-APETALA1* (*LAP1*) when overexpressed represses growth cessation and bud set in SD exposed plants (Azeez et al., 2014). Expectedly, *LAP1* RNAi constructs trigger early growth cessation and bud set in SD (Azeez et al., 2014, Ding and Nilsson, 2016). These results are very much like the functional analysis of *FT2* in *Populus*. On analysing the spatial expression patterns of *LAP1* it was found that it is expressed in the shoot apex and weakly in leaves. It was eventually shown that *LAP1* is in-fact the downstream element in the CO-FT photoperiodic pathway that controls growth cessation and bud formation phenology in Poplar (Azeez et al., 2014).

#### *FD*

FT that is expressed in the leaf's phloem cells is transported to the apex in LD where it acts as a promoter of vegetative growth. Since FT has no DNA binding domains it needs partner proteins that forms a DNA binding complex with it. FD is such a transcriptional factor belonging to the Basic Leucine Zipper Domain (bZIP) family which is also known to interact with other transcriptional factors in Arabidopsis (Abe et al., 2005). There are two *FD*-related homologs found in Poplar, *FD-like 1* (*FDL1*) and *FD-like 2* (*FDL2*) that encode proteins of 168-aa and 302-aa respectively. *FD* overexpression in Arabidopsis causes early flowering while *FDL1* and *FDL2* overexpression in Poplar show different phenotypes in comparison to WT. *FDL1* RNAi induced growth cessation and bud set while *FDL2* RNAi did not, suggesting that FDL1 is the important protein for FT interaction and affects bud phenology (Tylewicz et al., 2015). Apart from

its effects on bud phenology it is also shown to play an important role in adaptive response and bud maturation in short days (SD) along with Absciscic acid (ABA) via interaction with the ABSCISIC ACID INSENSITIVE 3 (ABI3) transcription factor (Tylewicz et al., 2015). FDL1 is able to bind to the promoter of LAP1 which acts as a connecting link with AIL1 and D-type cyclins thus regulating apical growth cessation and bud set. Neo-functionalization of FDL genes in hybrid aspen is due to structural differences between FDL1 and FDL2 although both FT1 and FT2 can bind to both proteins (Tylewicz et al., 2015).

### *AIL*

In order to identify genes downstream of Poplar FT for photoperiod regulated apical meristem growth cessation, (Karlberg et al., 2011) compared microarrays of apical buds of transgenic lines with WT (Karlberg et al., 2010). The data acquired identified *AINTEGUMENTA-like* genes along with cell cycle genes as main contenders. In Arabidopsis, *AINTEGUMENTA* is an AP2-domain containing gene that acts as a transcription factor in floral meristems and promotes flowering in long days (Karlberg et al., 2011). There are four homologs of *AINTEGUMENTA* in Poplar called *AINTEGUMENTA-like 1 to 4 (AIL1 to AIL4)* and are expressed in the shoot apical meristem and leaf primordia. On transcriptional fusion of a *GUS* reporter gene with the *AIL1* gene its expression was confined to zones of actively dividing cells in the apical meristem. Quantitative PCR expression data of *FT*- overexpressing Poplar plants that continue growing in SD conditions showed a higher expression of all four *AIL* genes, while, *FT* RNAi in SD had a loss of *AIL1* expression. This made them good candidates as downstream elements promoting vegetative growth. On overexpression of *AIL* genes it was found that the plants continued growing in SD identifying them as growth promoters. Later it was found that Poplar *LAP1*, was able to bind the *AIL1* promoter and regulate its expression (Azeez et al., 2014). *AIL* genes promotes the expression of D-type cyclins that are cell cycle genes by binding to their promoters (Karlberg et al., 2011). While downregulated *AIL* expression effected several other cell cycle genes including D-type cyclins culminating in cessation of growth and bud set.

### *Circadian clock*

Poplars are deciduous trees found in the temperate zone of the northern hemisphere. As there is a 23.4-degree tilt in the earth's axis there are seasonal changes to which life has to adjust. As we move towards the poles with increase in the latitudes the seasons become more pronounced. All organisms anticipate

events in the day in order to plan, optimize and regulate physiological and metabolic activity. This is important as across the 24 hrs period there is incredible change in environment i.e., day-night, temperature change. When seen in context of internal cellular processes, each metabolic cycle is interdependent and successive and must follow a sequence of events. It is vital that the dynamic environmental changes across the day are tied to the cyclical cellular events for the purpose of fitness. These cyclical events have certain regularly oscillating hubs. These hubs are genes that keep the clock and are called circadian clock genes (Eriksson and Webb, 2011). Disruption of this genetic network throws all the processes haywire and is thus central to fitness. The genes are not only required for day to day activity in organism but also vital to weave-in seasonal changes making the plants anticipate change and respond accordingly. Circadian clock is the recurrent oscillation of gene transcript's over a 24 hrs period. There are many genes having regular phases of peaks and troughs, but they have been largely divided into two elements. The Morning element with the two MYB transcription factors *LATE ELONGATED HYPOCOTYL 1* and 2 (*LHY1* and *LHY2*) and the evening element *TIMING OF CAB EXPRESSION 1 (TOC1)* (Takata et al., 2009). The *LHY1* and *LHY2* genes are expressed early in the morning with a progressive reduction in the evening. *LHY1* and *LHY2* have a feed-back loop of self-repression and also repress *TOC1* transcription. At the end of the day when *TOC1* expression comes up it represses *LHY1* and *LHY2* expression until morning. As the *TOC1* expression falls late in the night the morning elements are expressed. This cycle continues uninterrupted and thus acts as a regular timekeeper. The down regulation of the *LHY* and *TOC1* clock genes by RNAi results in an early growth cessation in *Populus* revealing their central role in regulating this process (Ibanez et al., 2010). We have recently shown that two *Populus* orthologs of the Arabidopsis circadian clock associated gene *GIGANTEA (GI)*, *PttGIGANTEA (PttGI)* and *PttGIGANTEA-like (PttGIL)* play important roles in photoperiod regulated apical growth cessation (Ding et al., 2018). *PttGI* is associated with the *PttFT2* promoter and regulates its expression (Ding et al., 2018). Apart from the core oscillators, *EARLY FLOWERING 4 (ELF4)* and *PSEUDO-RESPONSE REGULATOR (PRR)* family genes (Zawaski and Busov, 2014) regulate the circadian clock. Various Red/Far Red phytochrome family genes (*PHY*) and blue light chromophore receptors (CRY, FKF1, ZTL, LOV domain containing protein) feed into the circadian clock.

### *Hormonal regulation of bud phenology*

Hormones play an important role in shaping plant architecture and are difficult metabolites to study due to their ubiquitous presence and range of action. The most studied hormones in case of bud phenology are gibberellins (GA) and abscisic acid (ABA). Gibberellins are growth promotive hormones and are known to be upregulated in LD photoperiods (Eriksson et al., 2015). An exogenous application of GA promotes flowering and acts against growth cessation (Porri et al., 2012, Conti, 2017, Rinne et al., 2011). Poplar plants with higher expression of the GA biosynthetic GA20 oxidases do not cease growth in SD, while plants with higher expression of the catabolic GA2 oxidases cause early growth cessation (Eriksson et al., 2015, Singh et al., 2018a, Singh et al., 2018b, Tylewicz et al., 2018). Expression of GA in buds during the seasonal shifts is regulated indirectly by ABA. A higher ABA concentration with the advent of short days indirectly negatively regulates GA via the MADS-box gene *SVP-like* (*SVL*). This expression profile is similar to the hormonal profile in dormant seeds. Dormant seeds too have high ABA levels which is inversely proportional to GA, the only difference being lack of *SVP* expression in dormant seeds (Ruttink et al., 2007). This parallel suggests a redundant use of the same genetic and metabolic pathways to modulate different tissues and organs of the same plant. *SVL* is an ortholog of the Arabidopsis MADS-box protein *SVP*. *SVP* acts as a repressor of *FT* expression in Arabidopsis and its overexpression causes a delay in flowering (Hartmann et al., 2000, Andres et al., 2014). *SVL* is also related to the MADS-box protein genes *DORMANCY ASSOCIATED MADS* (*DAM*) in peach trees (Li et al., 2009). An overexpression of *DAM* genes results in an early growth cessation, bud formation and very late bud burst in peach trees (Li et al., 2009).

### *Plasmodesmata*

In actively growing meristems all the cells are connected by plasmodesmata, allowing cell-to-cell communication and a passage of signaling molecules and hormones (Paul et al., 2014). When Poplar plants are exposed to short days, they cease cell expansion, internode elongation and start forming apical buds. Transmission electron micrographs of apical cells in Poplar show formation of sphincters that block cell-cell communication. Recently, the role played by the ABA hormone in the formation of these blocking sphincters was shown (Tylewicz et al., 2018). When Poplar trees expressing a dominant negative gene construct (*abi1-1*) with reduced ABA sensitivity was compared to wild type trees, endodormancy was not established in the transgenic trees. Both WT and

*abil-1* mutants undergo growth cessation and bud formation on exposure to 4 weeks of SD. They were further exposed to a total of 11 weeks of SD and later re-exposed to LD. While, WT are endodormant and need cold treatment for re-initiation of growth, mutant lines burst buds and produced leaves on exposure to LD. The expression studies of genes regulated by ABA revealed upregulation of *GERMIN-LIKE 10*, *REMORIN-LIKE 1* and *2* (required for plasmodesmata function) and *CALLOSE SYNTHASE 1* (required for callose deposition) in WT, and downregulation of GH17-39 glucanases (implicated in sphincter removal) (Tylewicz et al., 2018). This suggested the involvement of ABA in maintenance of bud dormancy and bud set by mediating a short photoperiodic response with plasmodesmata closure. A further investigation by transmission electron micrographs shows a substantial reduction of plasmodesmata sphincters in mutant lines (Tylewicz et al., 2018). Plasmodesmata acts as a barrier by limiting access to growth promotive signal like FT. This was showed by grafting SD-induced WT and *abil-1* mutant scions to *FT1*-over expressing stocks. The mutant lines, due to lack of plasmodesmata sphincters, were able to burst leaves while the WT stems remained endodormant (Tylewicz et al., 2018).

### *Aging*

Trees are perennial in their life cycle so just like humans their growth is associated with incredible morphological changes over the years, also called as heteroblasty. The young juvenile plants prioritize their resources to grow vegetatively and gain enough biomass so that they could ensure their survival. This phase is called the juvenile phase that is characterized by the lack of reproductive features like flowers, fruits and seeds. In case of *Populus*, the juvenile plants are also distinct in the shape and size of their leaves and the distribution of trichomes and hairs on the abaxial and adaxial surfaces of their leaves and stem. These phases gradually change over years and is dependent on the *Populus* species (Wang et al., 2011). During the transition from the juvenile to the reproductive phase plants are also known to carry characteristics of the two phases together. There are many known examples when a single branch of the plant consists of both these phenotypes pointing towards the dynamic nature of this process. Plants like *Acacia* can have a single leaf showing both juvenile and adult characters. This is also seen depending on the position of the plant tissues, as older more basal plant tissues retain the juvenile characteristic while the newer tissues nearer to the apical meristems have adult characters (Wang et al., 2011). It is of interest for people studying plant developmental biology to decipher the genes and pathways involved in this phenotypic distinction. Due to evolutionary conservation in most plant genetic and developmental processes,

knowledge gained from studying *Arabidopsis* has been used as a steppingstone. It is now known that the main molecular players that are conserved between *Arabidopsis* and *Populus* also play a role in regulating plant vegetative and reproductive phase changes. On studying genetic data derived from trees like *Acacia* etc, we now know that, like in *Arabidopsis*, with increasing age, there is a gradual change in the expression of microRNAs in trees.(Wang et al., 2011) Since many different tree species belonging to the Angiosperm lineage show the same pattern, we can confidently say that the microRNA regulation of the juvenile-to-adult phase change is a strong and recurrent feature. Microarray analysis suggest that there is change in expression of the microRNAs miR156 and miR172 with the change in phase of the plants. In juvenile leaves, miR156 has a robust expression that decreases with age. miR156-overexpression in *Populus nigra* led to a retention of juvenile characters. However, heteroblasty is not that visually obvious in this species.

In *Populus*, the juvenile plants have trichomes on leaf and stem surfaces and large and smooth margined leaves while the adult plants display a gradual reduction of these trait. This phase transition varies temporally within annuals and perennials and is also species dependent, but the genes involved are evolutionarily conserved. Presently, miRNA156 and miRNA172 and their targets, the *SPL* and *AP2*-like transcription factor genes have been implicated. There are 11 miR156-encoding genes in *Populus* (Reinhart et al., 2002). Each subtype has a temporal and spatial expression profile with unique target accessibility. However, all miR156 subtypes when overexpressed are capable of downregulating the target *SPL* genes and affect phase change. Contrary to the miR156 expression pattern, miR172 has a low expression in juvenile plants that progressively increases in adult plants.

## 1.10 Bud Set or Bud Establishment and Dormancy

There are three types of dormancy as defined by Lang in 1969 and later elaborated in various reviews (Ruttink et al., 2007, Rohde and Bhalerao, 2007, Singh et al., 2017). i.e., ecodormancy, paradormancy and endodormancy. Ecodormancy is when the buds are formed, and the tree appears to be dormant. However, if the environment returns to conditions that is permissive to growth, the buds would flush, and growth would ensue. There are two variations of ecodormancy, one is formed during SD-triggered autumn bud set prior to a much deeper endodormancy and the other is post cold treatment when the endodormancy is overcome but the buds do not burst due to inclement weather (Singh et al., 2017). Both these stages of ecodormancy have a different global

gene expression and metabolic profile(Karlberg et al., 2010, Howe et al., 2015, Ruttink et al., 2007). Paradormancy is when axillary buds are maintained due to dominance of the apical shoot. Hormonal regulation is thought to play a major role in paradormancy as pruning the apical meristem frees lateral buds from dormancy (Rohde and Bhalerao, 2007). Endodormancy is when the shoot apical meristem does not reinitiate growth in permissible conditions (Ruttink et al., 2007, Rohde and Bhalerao, 2007). The plant has to undergo a cold treatment i.e., winter exposure for buds to burst and grow vegetatively. There are no molecular markers known yet that quantify this process, but one can speculate that *Populus* FT1, EBB, ABA, SVL, RCARs/PYL, and NCED3 (a key ABA biosynthesis enzyme) are good candidates. The dormancy mechanism might have close parallels with seed dormancy and germination, the difference being seeds have dividing cells while buds have expanding cells (Wellensiek, 1964). What adds to the fascination of this similarity is that seeds are believed to have evolved 100-400 million years after the buds. Traits found in seeds like chilling requirement to release dormancy, effects of the hormones ABA and GA on dormancy development and release, change in water availability and dissociation tolerance, accumulation of storage compounds could act as guide to deepen our understanding of bud phenology(Powell, 1987). There are several morphological, genetic and metabolic changes responsible for bud set, dormancy establishment and winter hardiness. All these processes are closely related and occur successively as day length decreases and temperature plummets with the advent of autumn and winter. It is very important that we understand that growth cessation, bud dormancy, bud set, and acquisition of hardiness are entirely different processes but many of their pathways are same or interdependent. Hormones like Gibberellins (GA) and Absciscic acid (ABA) regulate dormancy. Plants overexpressing GA do not set buds in short days (SD) suggesting that they are promoters of vegetative growth in trees (Eriksson et al., 2015). ABA levels build up in the apices in SD and are high in dormant buds. ABA insensitive *abil-1* mutants are known to not set buds properly in SD and the mechanism of regulation has recently been more clearly understood (Singh et al., 2018b). ABI1 is an absciscic acid receptor and the lack of its production disallows (*abil-1*) plants to enter endodormancy. *abil-1* plants exposed to 11 weeks of cold treatment after bud cessation and bud formation, re-start vegetative growth without cold treatment in warm SD conditions(Singh et al., 2018b). It is now understood that the plant requires ABA hormone perception to regulate the MADs box gene *SHORT VEGETATIVE PHASE (SVL)*. *SVL* is an ortholog of Arabidopsis *SVP* in *Populus*. *SVP* acts as a repressor of Arabidopsis *FT* expression by binding to its regulatory sites. The high *SVL* expression triggered

by ABA results in an upregulation of *CALLOSE SYNTHASE 1(CALS1)* and suppression glucanases that degrade callose. This results in callose deposition by CALS1 in intracellular conduits called plasmodesmata (PD). Plasmodesmata are intracellular conduits that allow the exchange of metabolites and signals between the plant cells. They are responsible for the transfer of signals originating in other parts of the plants to the apical meristems thus regulating its activity. It is thought that callosic plugs, also called “dormancy sphincters” that block the plasmodesmata conduits, cause isolation of the buds from rest of the plant tissues resulting in disconnection from growth promotive signals and promotion of endodormancy (Rinne et al., 2011, Tylewicz et al., 2018). SVL regulates the expression of the genes that cause the deposition of callose sphincters in the plasmodesmata and also negatively regulates the synthesis and perception of the growth promotive hormone gibberellin (GA) thus holding the endodormant state of the bud (Singh et al., 2018b, Tylewicz et al., 2018). Since ABA is also highly expressed in non-germinating seed in Arabidopsis it makes sense if similar kind of regulation is active in buds.

## 1.11 Cold Treatment and Bud Maintenance

As discussed earlier there are three known states of dormancy i.e.; ecodormancy, paradormancy and endodormancy (Rohde and Bhalerao, 2007). Cold treatment to release the endodormancy and turn the tree ecodormant is different from bud burst, as although buds may lose dormancy they may not burst and grow until warm spring arrives. The subtle difference between dormancy release and bud burst is important to predict the quantity and quality of cold treatment (chill-unit accumulation) required for different genotypes to lose endodormancy (Arora et al., 2003). Not much was known about the molecular players responsible to maintain dormancy until the recent characterization of the ABA/SVL module (Tylewicz et al., 2018, Singh et al., 2018a, Singh et al., 2018b), implicating the hormone ABA and its upregulation of SVL expression as affecting endodormancy establishment. SVL is an ortholog of Arabidopsis SVP which is known to be a repressor of FT expression (Hartmann et al., 2000, Fernandez et al., 2014, Liu et al., 2013). Plants that were ABA insensitive (*abi1*) or with repressed SVL expression, burst their buds without cold treatment in SD. It was earlier thought that a lack of promotive signal caused a state of stasis. But now this study shows how SD treatment shuts down the plasmodesmata conduits that are required for passage of metabolites that maintain vegetative growth, making the tree to enter endodormancy. It is possible, but has not yet been shown, that the endodormancy is then released because of the opening of the plasmodesmata



after cold treatment that is required to re-establish the connection between the apical bud and rest of the plant allowing intercellular transfer of metabolites causing vegetative growth (Tylewicz et al., 2018, Rinne et al., 2011, Paul et al., 2014).

## 1.12 Bud Burst

Trees survive the winter by suspending growth and forming apical buds to protect the meristem. But, in order to undergo bud-burst trees must first be exposed to cold treatment. Cold treatment re-establishes endogenous molecular pathways that lie suspended during the winter. There are a few contenders that could be established as marker genes to mark this change. Amongst them is the *FTI* expression level that incrementally increases in the buds depending on the time and intensity of exposure of trees to cold (Ding and Nilsson, 2016, Hsu et al., 2011, Pin and Nilsson, 2012). During bud burst the primordial leaves formed during the bud set that are covered by scales start re-growing and there is an internode expansion of the stem within the bud resulting in swelling and eventually into bud burst. The new leaves grow vegetatively completing the process. It is thought that bud burst is primarily temperature dependent. The mechanisms that are involved as the buds develop from cold treatment to bud burst have recently been discussed (Singh et al., 2018a, Singh et al., 2018b) and have implicated the role of the *SVL* gene. SVL regulates a web of hormonal ABA and GA pathways along with callose sphincter degradation to overcome endodormancy in buds. Exogenous applications of ABA and GA to trees triggers late bud burst and earlier bud burst respectively (Rinne et al., 2011, Rinne et al., 1994). This mechanism of action is very similar to the regulation of seed dormancy in *Arabidopsis* (Ruttink et al., 2007). Other studies have shown that the AP2/ethylene responsive factor domain containing *EARLY BUD BREAK1 (EBB1)* gene when overexpressed causes earlier bud burst, and when down regulated caused late bud burst, further reflecting the complexity of the event (Yordanov et al., 2014, Busov et al., 2016).

## 2 Objective

This thesis is based on three different lines of work. The first two manuscripts are the study of the ageing related microRNAs miR156 and miR172, and the third publication where I contributed was a study of effects of the gene *GIGANTEA* (*GI*) on *FLOWERING LOCUS T* (*FT*) expression in *Populus*.

### 2.1 Methodology

#### 2.1.1 Manuscript 1

miR156 overexpression in transgenic *Populus tremula* x *tremuloides* reveals novel aspects of miR156 regulation in trees.

##### *Plant material*

*Populus trichocarpa* and *Populus tremula* x *tremuloides* (T89) hybrid plants were used as model plants in the study. Since, the genomes of both the species are already published in the <http://popgenie.org> resource we have extensively used published transcripts and gene annotations from this resource. Leaves, buds, stipules and petioles were used for RNA extraction and comparative measurements during phenotyping.

In this project we have generated overexpression lines for miR156. The precursor gene was isolated and cloned from the genome of *Populus* and expressed from the Cauliflower mosaic virus (CaMV) 35S promoter. The *Populus* plants were transformed with this construct using *Agrobacterium*-mediated transformation of stem segments (Nilsson et al., 1992). The transformed plants were later studied for changes in their morphological and phenological characteristics. Since bud cessation, bud set, and bud burst were the traits of

interest, plants were subjected to day length shifts. Along with this, traits associated with the juvenility-to-adult transformation were also studied.

#### *Phenology observation and measurements*

The miR156 overexpressor plants were measured for height, distribution of trichomes on leaves, hairs on stem, cross section of petioles and size and length of leaves and stipules. The transgenic plants along with WT trees were first grown in a growth chamber under long day (LD 18hrs) conditions at 20-22°C temperature and 75% humidity. After measurements and sample collections the plants were shifted to short days (SD). There were two SD lengths used for two different batches of plants. One batch was shifted to SD 8hrs at 18-20°C at 75% humidity while another was shifted to SD 14hrs at 18-20°C at 75% humidity. The two different SD periods provided data with a hard SD shift and soft SD shift, respectively. The daylength shifts were based on the critical day length measurement for T89 plants i.e., 15.5 hrs (Olsen, 2010).

#### *Phylogenetic tree*

MicroRNAs are small 20-24 nt RNAs that act by targeting mRNAs that have a complementary target site. They act along with a family of targeting proteins forming an RNA Induced Silencing Complex (RISC). miR156 targets the *SQUAMOSA PROMOTER BINDING-LIKE (SPL)* family genes. This family contains 26 genes in *Populus*. A phylogenetic tree was constructed by alignment of protein sequences from all *Populus* and Arabidopsis SPL proteins by the MUSCLE algorithm and later using Maximum likelihood phylogenetic tree construction algorithm provided in the MEGA-X software program. The new *Populus* SPL proteins were annotated based on their similarity to the corresponding Arabidopsis SPLs.

#### *Cloning methods*

Selection of miR156 precursor genes to be overexpressed was based on the phylogenetic tree branching pattern of *Populus* sequences with that of Arabidopsis. *AtmiR156e* was found to be the most effective microRNA sequence in case of Arabidopsis in terms of causing phenotypic differences when overexpressed. *Populus PtmiR156c* and *PtmiR156e* branch together with *AtmiR156e* in a phylogenetic tree. We cloned and overexpressed both *PtmiR156c* and *Ptmir156e* under the control of the 35S promoter. Later, since

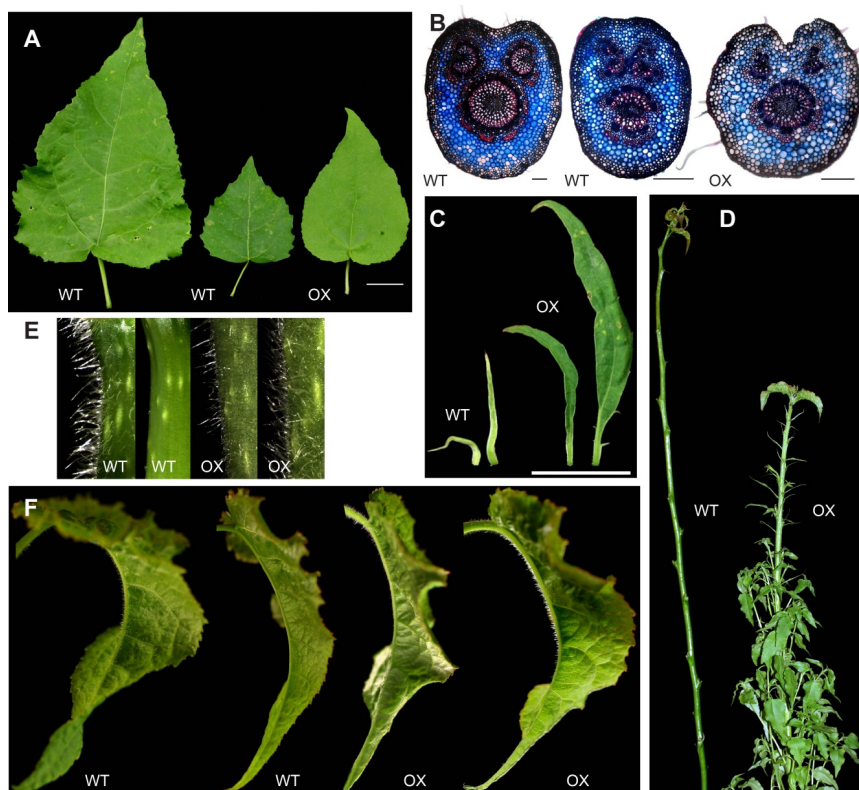
*PtmiR156e*-overexpression caused the most obvious phenotypic differences, we selected plants expressing it as the subject of our study.

### *Quantitative PCR*

Real time Q-PCR was performed on samples derived from *PtmiR156e* overexpressor transgenic lines and WT plants. Leaf samples were collected at the end of the light period in LD i.e., ZEITGEBER TIME (ZT) 16 to 17.

### *Results*

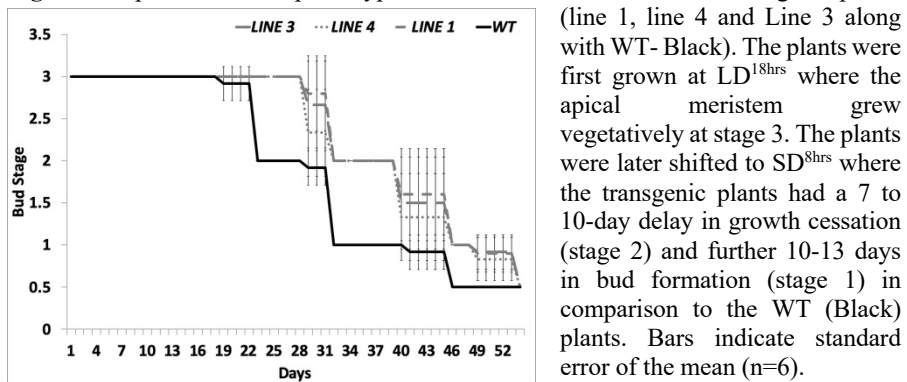
We found that transgenic plants in their first growing season were shorter in comparison to WT. The transgenic plants had a reduced plastochron and shorter internodes. They had lighter colored leaves that were larger in size (Fig. 3) in comparison to WT. The leaves were covered in trichomes. The cross sections of the petioles were heart shaped in the first year, while the stipules of the transgenic lines were larger in comparison to the WT (Fig.3).



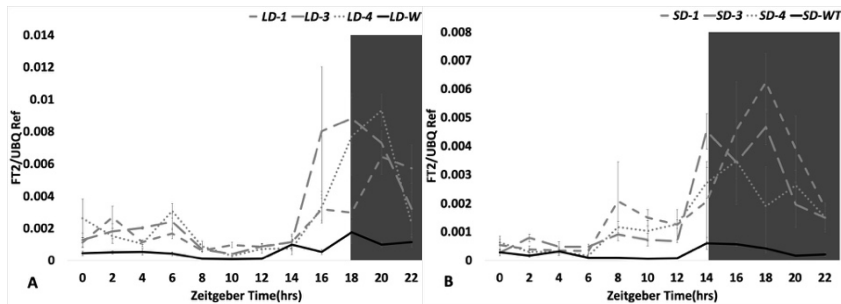
**Figure 3:** *35S::PtmiR156* phenotypes. **(A)** Completely expanded leaf from wild type *Populus tremula* x *tremuloides* (WT) from the first growth season followed by wild type and *35S::PtmiR156* (OX) leaves from short shoots in their second growing season. Scale bar represents 2 cm. **(B)** Cross sections of petioles from leaves at 30 cm height. Petiole from a fully expanded wild type leaf in its first growing season, about 1 month after potting followed by a petiole from wild type and *35S::PtmiR156* trees about 3.5 months into its second growing season. Scale bars represent 200 $\mu$ m. **(C)** Stipules, the two first from wild type and the second two from *35S::PtmiR156* trees in their first growing season, 2 months after potting. A representative and a large stipule for each genotype are shown. The scale bar represents 2 cm. **(D)** Top 1/3<sup>rd</sup> of wild type and *35S::PtmiR156* trees in their first growing season, three months after potting. The leaves have been removed to make the sylleptic branching and enlarged stipules more visible. **(E)** Stem from wild type (two first) and *35S::PtmiR156* trees (second two) in their first growing season. Both wild type stems, and the first transgenic stem shows the phenotype four weeks after potting, while the second transgenic stem shows the trichome phenotype one year after potting. **(F)** Two leaves from wild type and two from *35S::PtmiR156* trees in their first growing season. Both wild type leaves, and the first transgenic leaf shows the phenotype six weeks after potting, while the second transgenic leaf shows the trichome phenotype one year after potting.

Other observations were the smaller axillary buds formed on the transgenic lines in comparison to WT. The leaves during the second growing season were long and elongated in transgenic lines while they were short and rounded in the WT. The transgenic leaves were lighter in color and intermediate in size in comparison to first year transgenic and WT leaves. The second year WT leaves were darker in color and had no trichomes while the second-year transgenic leaves retained the trichomes. Taken together, this suggested a retention of juvenile phenotypes in the miR156 transgenic plants during the second growing season. On shifting the plants from LD to SD the transgenic plants set buds one week later in comparison to WT (Fig 4).

**Figure 4:** Apical meristem phenotype measurements for *PtmiR156* transgenic plants



On studying the expression profiles of the genes by quantitative PCR we found that the expression of PttmiR156 in the transgenic lines was higher than in WT during both the first and second growing season. There is a decrease in PttmiR156 expression in the second year WT plants in comparison to first year WT plants, similar to the case in Arabidopsis and Acacia plants (Wang et al., 2011). Since earlier research had described the photoperiodic regulation of the CO-FT module and its effects on bud phenology (Bohlenius et al., 2006) we checked whether miR156 had any role to play in modulation of *FT* expression and bud phenology. Accordingly, we shifted the transgenic and WT plants from LD 18hrs to SD 8hrs, which is a much shorter day length than the 15.5 hrs critical day length for the T89 clone (Olsen et al., 1997). We later measured the change in bud phenology according to (Ibanez et al., 2010). The transgenic lines cease growth and set buds almost 7 day later than the WT plants (Fig .4). This suggests that miR156 overexpression delays growth cessation and bud set in transgenic lines. This result is counter-intuitive as it suggests that growth promoting genes are upregulated and active. This was later confirmed by Quantitative RT-PCR expression studies of *PttFT2* expression. We found that the *PttFT2* expression was higher in both LD and SD of transgenic lines compared to WT (Fig 5)



**Figure 5:** Relative expression of *PttFT2* in diurnal samples from *Populus tremula x tremuloides*. The leaf samples were collected every 2hrs during a 24 hrs period. Expression of *PttFT2* in A) LD<sup>18hrs</sup> and B) SD<sup>14hrs</sup> is significantly higher in transgenic lines than in the WT (Black) at the end of the day. Bars indicate standard error of the mean (n=3).

## 2.1.2 Manuscript 2

Role of miR172 and its target AP2-like transcription factors in growth cessation and bud set.

### *Plant material*

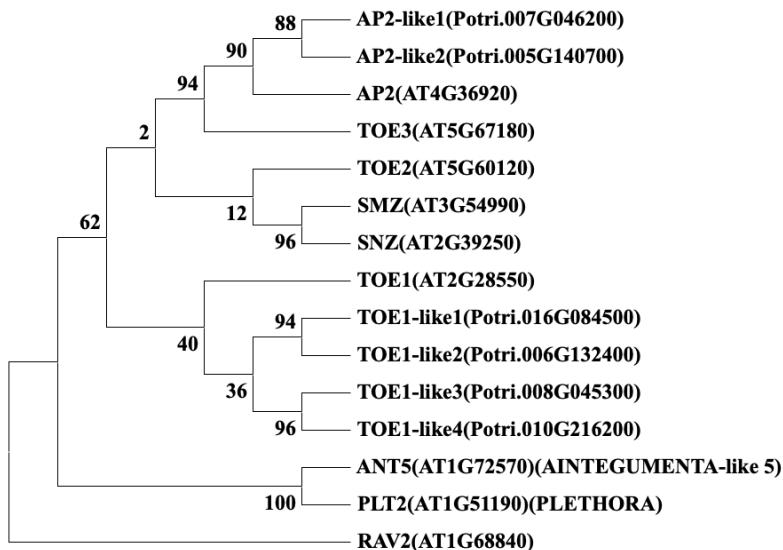
There are two approaches to do a comparative study of genetic traits in any model system by reverse genetics i.e., either you overexpress the gene of interest by a strong promoter or by downregulating the gene of interest. Both approaches have their pros and cons. Since, miR172 is a large family in *Populus* made of 9 miR172-transcribing genes with redundant functions both approaches can be useful. We isolated a miR172 precursor gene from the *Populus* genome and expressed from the 35S promoter in transgenic trees. The 10 calluses that we obtained during the transformation process were not able to shoot and survive on selection medium. We also generated a MIMICmiR172 construct and transformed it into hybrid aspen trees. Target mimicry technology allows the inactivation of all miR172 transcripts in the transgenic plants through the binding of a complimentary sequence that sequesters the endogenous miR172 transcript, thus making it inactive (Franco-Zorrilla et al., 2007). The transformed plants were grown in LD18hrs at 20-22 °C temperature and 75% humidity in a growth chamber. They were shifted to SD14hrs at 18-20°C temperature at 75% humidity. Diurnal leaf samples were collected in both LD and SD over a 24hr period. The SD samples were collected 7 days after shifting to SD conditions for subtle detection of change in expression levels of the transcripts due to changed photoperiod. We also generated overexpression constructs for the AP2-like transcription factor genes in *Populus*. The AP2-like transcription factors are targets of the miR172-RISC complex and their expression is downregulated in plants with higher miR172 expression (Wang et al., 2011). On phylogenetic analysis with known AP2-like transcription factors that are miR172 targets in *Arabidopsis*, like TARGET OF EAT1(TOE1), TOE2, SCHLAFMÜTZE (SMZ), SCHNARCHZAPFEN (SNZ) and APETELA2 (AP2), with their homologous sequences in *Populus tremula* x *tremuloides* we found 6 homologous genes. We cloned these genes and later generated miR172 resistant genes. The resistant genes were attached with a prefix (r) and called *rTOE1-like1* (*rTOL1*), *rTOE1-like3* (*rTOL3*) and *rAP2-like1* (*rAP2L1*). These cloned genes were later introduced in an overexpression construct called p2GW7 which contains the 35S promoter. The overexpression transgenic lines generated (Nilsson et al., 1992) were used in LD to SD shift experiments similar to those described for the MIMIC lines above. They were also phenotyped and their leaf

samples were collected for gene expression analysis in the same manner. After shifting the *35S::MIM172*, *35S::rTOL1*, *35S::rTOL3* and *35S::rAP2L1* lines to SD they were thoroughly measured for height, size of leaves and bud phenology. We used the LIACA machine to measure the size of the leaves in both transgenic and wild type plants. To complement our results from the *35S::rTOL1*, *35S::rTOL3* and *35S::rAP2L1* transgenic lines in *Populus tremula* x *tremuloides* we also generated transgenic lines with the same constructs in the *Arabidopsis thaliana* Columbia genotype. The transgenic lines were generated by the floral dip method (Clough and Bent, 1998)

### *Phylogenetic tree*

The miR172 RISC complex targets transcripts from AP2-like transcription factor genes in Arabidopsis. This is because these transcripts contain complementary target sequences to the miR172 sequence. There are 6 AP2-like transcription factor genes in Arabidopsis that act as repressors of flowering when overexpressed (Zhu and Helliwell, 2011, Mathieu et al., 2009). Their mode of action is via binding to the promoters of flowering activator genes like *FT*, *SOC1* etc (Aukerman and Sakai, 2003, Huijser and Schmid, 2011). Downregulating the activity of miR172 by target mimicry promotes the expression of these repressors in Arabidopsis, resulting in delayed flowering. Since we were interested in the miR172 mode of action in *Populus* it was natural to search for AP2-like transcription factor genes in the *Populus* genome. We found 6 sequences that had AP2 domains and that also contained targeting sites for miR172. To determine their relationship to the Arabidopsis genes we performed a phylogenetic tree analysis. This was done with the help of the MEGAX software (Kumar et al., 2018). The *Populus* and Arabidopsis sequences were first aligned with a MUSCLE algorithm and then run in a Maximum likelihood algorithm, Tamura 3-parameter method, with invariant sites and partial deletion (50%) and 1000 bootstraps resampling's. (Fig.6). The 6 genes that were found in *Populus* grouped in 3 pairs and were annotated according to the Arabidopsis sequences with which they branched. They were named *TOE1-like1* (*TOL1*) (Potri.016G084500), *TOE1-like2* (*TOL2*) (Potri.006G132400), *TOE1-like3* (*TOL3*) (Potri.008G045300), *TOE1-like4* (*TOL4*) (Potri.010G216200), *AP2-like1* (*AP2L1*) (Potri.007G046200) and *AP2-like2* (*AP2L2*) (Potri.005G140700).





**Figure 6:**Phylogenetic reconstruction of the AP2 domain containing families in *Arabidopsis* and *Populus trichocarpa* A phylogenetic tree was generated with the MEGAX software (Kumar et al., 2016, Kumar et al., 2018) based on codon sequence alignment (MUSCLE) (Edgar, 2004), Maximum likelihood, Tamura 3-parameter method with invariant site (Tamura, 1992), partial deletion (50%) and 1,000 bootstraps re-sampling. Genes were named based on their similarity with the Arabidopsis genes in the phylogenetic tree.

### Cloning methods

The MIMIC miR172 constructs were provided to us by Prof. Detlef Weigel and were transformed into the *Populus tremula* x *tremuloides* clone T89 (Nilsson et al., 1992) The MIMIC miR172 constructs were first used to transform *Arabidopsis* plants and their downstream target expression and plant phenotype was studied as described in (Franco-Zorrilla et al., 2007, Todesco et al., 2010). We also constructed miR172-resistant forms of *TOL1*, *TOL3* and *AP2L1*. The resistance was introduced in the cloned gene by replacing the miR172 complementary nucleotide sequence with other nucleotides while maintaining an intact codon degeneracy. This means that although the transcribed gene sequence is different from the WT, the translated protein is the same. This disallows the miR172 to sequester the cloned resistant sequence or degrade it. The *rTOL1*, *rTOL3* and *rAP2L1* sequences were fused to the ubiquitously expressed CaMV- 35S promoter. The same constructs were also transformed

into Arabidopsis plants to study their effects on the flowering phenotype since AP2-like transcription factors act as repressors of *FT* expression

### *Quantitative PCR*

Diurnal leaf samples were collected from all the transgenic lines that were involved in our experiments. We selected three transgenic lines per construct and collected samples from the 10-12th leaf counted from the apex every 2 hrs diurnally. The leaf samples were collected under both LD and SD conditions where the SD samples were collected 7 days after the shift. We used green fluorescent light to collect samples at night.

### *Results*

We first compared the *MIMI72* transgenic lines with WT plants in both LD and SD. All 3 transgenic plant lines were shorter than the WT and had a reduced plastochron and with shorter internodes. The leaves were larger in comparison to the WT and curly like as seen with the miR156-overexpression trees in Manuscript 1. After defoliation the axillary buds were exposed and found to be smaller than the WT buds. This trait is also reminiscent of miR156 overexpressor lines from Manuscript 1. We later shifted the plants from LD to SD conditions and measured the growth cessation and bud formation of the plants (Ibanez et al., 2010). The transgenic plants ceased growth and set buds one week later than the WT plants. This was unexpected and counter intuitive to the Arabidopsis model, since the activity of the miR172 target AP2-like transcription factor genes should have been increased as a result of the sequestering and being repressors of *FT2* expression should have resulted in early growth cessation in the transgenic plants. We then checked the expression of *FT2* by Q-PCR in both transgenic and WT plants in LD and SD. We found that *FT2* was expressed at significantly higher levels in *MIMI72* lines in comparison to WT. This consistent with the late growth cessation seen in the *MIMI72* trees. The next step was to phenotype the miR172-resistant AP2-like transcription factor transgenic lines in LD and SD conditions. All transgenic over-expressor plants were strikingly shorter than the WT plants. The leaves of all transgenic plants were curly like in the *MIMI72* lines. On studying the growth cessation and bud formation of transgenic lines we found that except for the *rAP2L1* overexpressor lines all other transgenic plants set buds at the same time as WT. The *rAP2L1* overexpressor plants cease growth and set buds much later than WT plants. We checked the putative downstream target genes of AP2L1 i.e.; *FT2* and *LAP1*. Both *FT2* and *LAP1* expression was upregulated in both LD and SD in transgenic lines. This

expression profile of these important growth promoting genes is similar to the results in the *MIM172* lines. We had also transformed the *rTOL1*, *rTOL2* and *rAP2L1* overexpressor constructs into the *Arabidopsis thaliana* Columbia genotype. We checked the phenotype of these transgenic plants in both LD and SD. There were no flowering time effects in LD, but plants subjected to SD flowered much earlier than the WT controls. This result matches our results from the transgenic *Populus* lines as it suggests that the *Populus* AP2-like transcription factors are promoters of *FT* expression rather than being repressors. Earlier, *EARLY BUD BREAK1* (*EBB1*), another AP2-domain containing gene has been designated as a promoter of bud burst in trees (Busov et al., 2016, Yordanov et al., 2014)

### 2.1.3 Combined Discussion of Manuscript 1 and 2

MicroRNAs are small non-coding 20-24 nt RNAs that regulate gene transcription by a complementary binding to messenger RNA to regulate its function. Generally, most microRNAs bind the complementary target site present in the mRNA and degrade it. This works by a mechanism of combination of miRNA and a suite of proteins that form an RNA-induced silencing complex (RISC). The two most studied miRNAs are the age and phase determining miR156 and miR172. Both these miRNAs have been well studied in the *Arabidopsis* model and have been implicated in modulating the juvenility-to-maturity transition in terms of the regulation of flowering and vegetative phase change. They are found in all Angiosperm plants and seem to have the similar spatial and temporal expression patterns in all species. There are some unique modes of transcript repression, as in miR172 the repressive mechanism is not only through degradation of its target sequence but also by sequestration, inhibiting translation. The miR156 is highly expressed in seeds and young juvenile plants and its expression decreases with the age of the plant. In contrast, miR172 expression rises incrementally with the age of the plants. miR156 targets the *SQAMOSA PROMOTER BINDING LIKE* (*SPL*) family of genes, while miR172 targets the *APETELA2* (*AP2*) – like transcription factor family of genes (Huijser and Schmid, 2011). The *SPL* genes act as promoters of floral meristem identity genes in *Arabidopsis* while the AP2-like transcription factors like TOE1, TOE2, TOE3, AP2, SMZ and SNZ act redundantly as repressors of floral meristem identity genes like *LFY*, *API*, *LFY* and *FT* (Mathieu et al., 2009, Wu et al., 2009). The miR156 expression also effects the expression of *SPL9* that in turn regulates miR172 transcription by binding to the promoter of its precursor gene (Wu et al., 2009). This suggest that there are feedback loops

operational in the miR156-SPL-miR172-AP2 pathway that fine-tunes the entire process (Wu et al., 2009). In manuscript 1 we have done a comparative study of age, juvenile-to-vegetative phase change, growth cessation and bud phenology of *Populus tremula* x *tremuloides* by overexpressing miR156. We found that overexpressor plants are shorter than the WT with a curly leaf phenotype and axillary buds much smaller than WT plants. This phenotype is similar to the transgenic *MIMmiR172* and AP2-like transcription factor overexpressor plants in manuscript 2. Both transgenic plants from manuscript 1 and manuscript 2 also follow the same pattern of growth cessation and bud set phenology when shifted from LD to SD conditions. On conducting expression studies of putative downstream targets in transgenic lines by Quantitative RT-PCR in both manuscripts, we found the expression of *FT2* to be upregulated in both LD and SD condition. This established an emergent pattern of gene expression and phenotype of the plant. What was even more intriguing is the counter intuitive expression of *FT2* in both manuscripts. In case of Manuscript 1, miR156 targets *SPL* genes that are activators of flowering in Arabidopsis and usually associated with a higher *FT* expression, and in Manuscript 2 MIMIC miR172 downregulates the activity of AP2-like transcription factors that in Arabidopsis normally act as repressors of *FT* expression (Fig. 7). Both these results suggest that the miR156-SPL-FT2 and miR172-AP2-FT2 modules are wired differently in the case of *Populus*. If we look holistically, the results that both miR156oe and *MIMmiR172* lines have similar phenotypes and gene expression seems logical. While miR156 is high in young juvenile plants its overexpression should hypothetically produce the same results as a MIMICmiR172 line, as miR172 has a reduced function in these lines. miR156 and miR172 expression being inversely proportional in nature gives logical support to the phenotypes in both transgenic lines. A possible explanation to the discrepancy between the Arabidopsis and *Populus* models could be attributed to a difference in the function of the AP2-like transcription factors. When we overexpressed miR172-resistant constructs of the *Populus* genes in Arabidopsis they promoted flowering and *FT* expression, rather than repressing it as do their Arabidopsis homologs. Likewise, the overexpressors in *Populus* triggered late growth cessation and increased *FT2* expression. Taken together, this suggests that the *Populus* AP2-like transcription factors have evolved into activators of *FT* expression. Furthermore, this suggests that the increased *FT2* expression found in miR156 overexpressors could be attributed to a reduced expression of *SPL* genes leading to lower expression of miR172 thereby increasing the activity of the *FT*-activating AP2-like transcription factors (Fig. 7).



*PttFToe* lines (Bohlenius et al., 2006). On conducting the photoperiodic day length shift experiments we found that the WT plants cease growth after 4 weeks in SD10h and set buds after 9 weeks while the overexpressor lines cease growth one month later than WT. This suggests that the transgenic lines are less sensitive towards changes in photoperiod. We also downregulated the expression of both *GI* and *GIL* paralogs by RNA interference (RNAi). A single construct targeted both *GI* and *GIL*. The growth cessation response for the *PttGIRNAi* lines was much stronger than what was seen in RNAi lines down-regulating the *PttGI* downstream targets *PttFT* and *PttCO* (Bohlenius et al., 2006). All the transgenic lines we produced had to be grown in LD23h because shifting them to shorter daylengths caused growth cessation. The bud phenotype of the transgenic lines was similar to WT suggesting that the importance of *PttGI* in regulating growth cessation is separate from the regulation of bud development.

### *Quantitative PCR*

Both RNAi and overexpressor transgenic lines gave consistent results on the importance of *GI* and *GIL* in growth cessation and bud set. Since, *CO* and *FT* are downstream targets of *GI* in Arabidopsis, we checked their gene expression profile by quantitative RT-PCR. There are two *FT* homologues in *Populus*, *PttFT1* and *PttFT2*, and similarly there are two *CO* homologues, *PttCO1* and *PttCO2*. *PttFT1* and *PttFT2* have different spatio-temporal expression patterns. While *PttFT2* is highly expressed during vegetative growth in *Populus*, *PttFT1* is incrementally expressed in apical buds of the plants when exposed to cold temperatures during winter. On checking *PttFT2* expression in *PttGloe* and *PttGILoe* plants, we found it to be 5-fold overexpressed compared to WT plants. This explains the continuous growth of transgenic plants in SD and a late growth cessation and bud set in SD10h. Surprisingly, *PttCO1* and *PttCO2* expression in transgenic lines was not that different from the WT plants in *PttGloe* and *PttGILoe* trees. In *PttGIRNAi* plants the *PttFT2* expression was almost abolished, while the peak expression of *PttCO1* and *PttCO2* was reduced during both the morning and evening peaks. This suggests that, unlike Arabidopsis where the CO-FT module is central to the regulation of FT and consequently flowering, there is an important CO-independent pathway involved in *Populus* FT regulation. We therefore used chromatin immunopurification (ChIP) assays to explore the possibility of *PttGI* directly interacting with the *PttFT2* and *PttCO* regulatory elements to regulate their expression. We found that *PttGI* indeed interacts with the 5'-prime UTR and 1.5 and 2kb upstream promoter regions of *PttFT2*, and also with a region 3kb upstream of the *PttCO2* translational start site. This shows the potential for a CO-independent regulation of *FT*, but also

suggests a direct role of PttGI in the regulation of *PtCO*, as is the case in Arabidopsis. Since GI is not a transcription factor, we believe that these bindings are indirect through an interaction with transcription factors like CDFs (see below)

#### *PttGI interaction with PttFKF1 and Ptt CDF*

In Arabidopsis, CDFs are transcriptional repressors that bind to both *CO* and *FT* promoters to repress them. This repression is countered by the GI and FKF1 co-expression that binds to the CDFs and degrades them in blue light (Song et al., 2015). ChIP analysis has shown that all three types of protein i.e.; GI, FKF1 and CDFs, bind to the promoter of *FT* and regulates flowering. We were interested whether the same process is also involved in the regulation of the CDF repressors in *Populus*. On analysing the *Populus* genome we found two *FKF1*-like genes in *PttFKF1a* and *PttFKF1b*, and eight *CDFs*, four of whom grouped together with known Arabidopsis repressors of flowering and were called *PttCDF1*, *PttCDF2*, *PttCDF3* and *PttCDF4*. In order to decipher the protein-protein interactions of PttGI, PttGIL, PttFKF1a, PttFKF1b and the four PttCDFs we conducted yeast two hybrid assays. Both PttGI and PttGIL showed strong interactions with PttFKF1b but not with PttFKF1a. PttGIL interacted with all four PttCDFs while PttGI more strongly interacted with PttCDF1. In case of PttFKF1a, it interacted with PttCDF1 and PttCDF4 while PttFKF1b interacted with PttCDF1 and PttCDF2. On further performing bimolecular fluorescence complementation assays (BiFC) in tobacco with the same set of proteins, we found that, in plant nuclei *in vivo* PttGIs can interact with both PttCDF1 and PttFKF1b and also that PttCDF1 and PttFKF1b can interact with each other. Furthermore, the differential interactions shown by PttGI and PttGIL with PttCDFs suggest that PttGI and PttGIL may have different functions in *Populus*.

#### *Functional study of CDFs in Populus*

As *Populus* CDFs were similar to Arabidopsis CDFs in their interaction with PttGIs and PttFKF1, we decided to study their function in *Populus*. We overexpressed *PttCDF3* by using the same vector as used for overexpression of *PttGIs*, *PttCO* and *PttFT2*. We later tested these transgenic lines in photoperiod regulated growth chambers for effects on growth cessation and bud set. While the WT plants took 4 weeks to cease growth and 8 weeks for bud set, the *PttCDF3oe* lines cease growth quickly and set bud in 6 weeks after shifting from LD18h to SD14h. This suggests that the transgenic lines were more sensitive to the shift in photoperiod than WT. The expression of *PttCO2* and *PttFT2* in the

transgenic lines was much reduced in comparison to WT in LD18h. We then generated *PttCDFRNAi* lines that down regulate the expression of both *PttCDF3* and *PttCDF4* and found them similar to WT in bud phenotypes and bud set. This suggests that there might be a redundancy in the function of the 8 *Populus* CDFs. Later, after conducting electrophoretic mobility shift assays (EMSA), we indeed found PttCDF binding to putative DOF-binding sites (AAAG/CTTT) in the 5'-UTR of *PttFT2*

### *Discussion*

The GI-CO-FT module is central to the photoperiodic control of flowering time in Arabidopsis. GI overexpression in Arabidopsis plants leads to a strong upregulation of both *CO* and *FT* (Mizoguchi et al., 2005, Suarez-Lopez et al., 2001). Likewise, overexpression of *CO* strongly induces flowering time via an induction of *FT* expression (Suarez-Lopez et al., 2001). Loss-of-function mutations in *CO* leads to reduced *FT* expression, and both *co* and *ft* mutants are to a similar extent late flowering (Bohlenius et al., 2006). This suggests that the CO-FT module is central to Arabidopsis flowering and that the GI effects on *FT* expression is mainly mediated through CO. Although the same set of genes are also present in many angiosperms that have been studied, not much is known about the relative importance of *FT* regulation by CO-dependent and CO-independent processes. We in our analysis of *Populus* genome sequences have identified two *GI*-like genes we call *PttGI* and *PttGIL*. Our diurnal expression analysis of both *PttGIs* found them to have similar expression profiles over a 24 hr period, and similar to Arabidopsis *GI*, with *PttGIL* having a weaker amplitude. We found that overexpression of *PttGI* and *PttGIL*, although causing late growth cessation and bud set in *Populus*, did not affect the expression of *PttCO1* and *PttCO2* as dramatically as was expected. We found that PttGI can directly interact with *PttFT2* upstream sequences, suggesting a CO-independent regulation of *PttFT2*. This is further supported by comparing RNAi-lines that downregulate the expression of both *PttCO1* and *PttCO2* with those down-regulating *PttFT* (Bohlenius et al., 2006). Compared to the *PttFT* RNAi lines, the *PttCO* RNAi lines have a much weaker effect on SD-induced growth cessation, and also affect the expression of *PttFT* to a much lower extent (Bohlenius et al., 2006). This again shows that PttCO has a minor role in the regulation of *PttFT* compared to the effects of Arabidopsis CO on *FT* and that the CO-independent regulation of *PttFT* expression by *PttGI* might be much more important. Later, ChIP experiments and EMSAs showed that PttGIs and their interactors PttCDFs are recruited to the *PttFT2* genomic region suggesting a mechanism for how PttGIs can regulate the *PttFT2* expression directly. We



also studied the functional conservation and divergence between the *PttGI* and *PttGIL* genes. We found that both genes when overexpressed cause sylleptic branching, which is an effect similar to what is seen in *PttFT2oe*. Arabidopsis GI is known to be involved in a variety of different physiological processes which includes flowering time regulation, light signaling, hypocotyl elongation, and abiotic tolerance (Mishra and Panigrahi, 2015)

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## Acknowledgements

This has been a long journey, but I learnt a lot thorough this experience. So, thank you Professor Ove Nilsson for providing me with the opportunity to work in your lab. Thanks to my reference group Thomas, Maria, Catherine for being so supportive, encouraging and giving me inputs from time to time. Then I would like to thank the Swedish people for allowing me to stay in your beautiful country and providing the resources for this undertaking. There are many others that have been with me for so many years.

The first people that come to my mind are my parents that have been supportive for all these years. Thanks to my sister Prajakta for being there for me in thick and thin. Thanks to Amol and loads of love to Little Aditya and baby Advait, you guys are just amazing, and I look forward to meeting you soon.

Thanks to Raja Kaka, Rishi dada, Rupali vahini, for all the encouragement. Thanks to Tanushree Sai and Ashwini. Thanks to Sanju dada for introducing RESEARCH to me. Apart from that I would like to thank all the friends I made in Umeå. The Great Maratha Mandal members Shrikant, Pratibha, Tushar, Abhilasha, Anjana, Dilip, Amol for all the good memories. Thanks to Prashant with whom I spend such great time. Thanks to Sonali for being such a good friend and Sachin for being such an inspirational person, there was not a moment Sonali does not talk about your great effort in vulture conservation. Thanks to Dharmesh for the long friendship and still being in touch. Thanks to Rahul for being a good friend. Thanks to Cuong and Beata for the open and frank conversations.

Thanks to all my Office mates over the years especially Alfonso Mateo for all nice conversation and it's all coming true when we look around. I would also

like to thank my Ove Nilsson group mates over the years Maria, Pierre, Dominique, Melis, Bo, Alfredo, Mark, Jihua, Keh Chien, Alice, Daniela, Louise. Thanks to the Indians at UPSC Sanat, Aparna, Rajesh, Vikas, Amit, Shashank. Thanks to Ingela, Inga-lis, Ingabritt, Veronica, Ann, Simon, Jan, Thomas for always being helpful. Thanks to the entire greenhouse staff past and present for taking care of my plants. Thanks to Bernard and Gosia for being the warm and welcoming personalities you guys have been. Azul to Abdullah. Thanks to Amir for the nice discussions. Thanks to Kristopher for all the nice conversations. How can I forget Stefana, Valentina and Titti! Also, thanks to Ionea, Chanaka, Haleh, Pali, Anne, Marta, Alexander, Jean Claude, Anita, Slim, Junko, Pieter, Junko, Barbora, Evgeniy, Ilara, Sacha, Nicolas, Ruben, Chen, Peter, Bibek, Thomas, Sara, Hong, for the smiling faces. And finally, I thank GOD for everything.

